

THE MITOCHONDRIAL ADENINE
NUCLEOTIDE TRANSLOCATOR
FROM *ZEA MAYS L.*
GENE STRUCTURE AND EXPRESSION

BY

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I declare that this thesis was written and composed by myself, and that the data contained within are my own, unless otherwise stated.

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ABSTRACT

To understand the molecular genetic basis of mitochondrial biogenesis during plant development, and the regulation of oxidative phosphorylation in particular, several nuclear genes encoding maize mitochondrial proteins had previously been cloned. Two of these genes termed *ANT1* and *ANT2* encode mitochondrial adenine nucleotide translocators. The work presented describes further characterisation of the *ANT* genes and isolation of a cDNA clone for *ANT2*. Steady state levels of *ANT* transcript have been analysed in different maize tissues and compared with other nuclear and mitochondrial genes encoding subunits of ATP synthase.

The *ANT* genes have at least 5 exons termed exon 1 to exon 5 and at least 4 introns termed intron A to intron D. The 5' end of the gene is more complex than originally concluded (Bathgate *et al.*, 1985). Both *ANT* genes contain multiple transcript initiation sites, but the position and number of sites are not conserved between the two genes. The sizes of the 5' untranslated regions (5' UTRs) are between 124 bp and 356 bp as predicted from the various primer extension products. Both *ANT* genes have a minimum of two untranslated exons and two large introns (intron A and intron B) separate the untranslated exons. The 5' and 3' splice sites of intron A have not been accurately determined for *ANT1* or *ANT2* but the size of the intron in *ANT2* is in excess of 2.4 kb. Intron B is 1063 bp and 1021 bp in size in *ANT1* and *ANT2* respectively and is separated from intron A by exon 2 which is approximately 85 bp and 78 bp in *ANT1* and *ANT2* respectively. The start of translation is in exon 3 and is 21 bp 3' of the intron B 3' splice site. Sequence of the *ANT2* cDNA clone confirmed that the open reading frame includes a presequence which has been reported to be cleaved following import into mitochondria (Winning *et al.*, 1992).

ANT transcript steady state levels vary in a tissue specific manner. The highest steady state transcript levels were found in tissues known to exhibit mitochondrial oxidative phosphorylation and high metabolic rates. Tissues with lower metabolic rates or those which are photosynthetically active contain lower transcript levels, hence, a tentative correlation has been made between the transcript steady state

level and metabolic activity. Probes which differentiate between the two ANT genes were used to investigate the possibility of differential regulation. *ANT2* transcripts have been shown to have a 3.5 fold range between different tissues whereas the range of *ANT1* transcript levels were indirectly estimated to be greater than 10 fold. Another maize gene encoded in the nucleus, the β subunit of ATP synthase, was shown to have a similar expression pattern to the ANT genes studied. This observation may indicate that their expression is coordinated which could be a consequence of both proteins being essential for oxidative phosphorylation.

Expression analysis of two mitochondrial genes, *ATP9* and α subunit, both subunits of ATP synthase, reveals much less variation of transcript levels between the different tissues. This suggests there is no coordination at the level of transcription between nuclear and mitochondrial genes encoding subunits from the same protein complex.

ABBREVIATIONS

3'	3' carbon of a nucleotide or nucleoside, hydroxy terminus of a nucleic acid backbone, towards the 3' carbon
5'	5' carbon of a nucleotide or nucleoside, hydroxy terminus of a nucleic acid backbone, towards the 5' carbon
5' end	5' end of a gene
5' UTR	5' untranslated region of a gene
A	adenosine nucleotide
AdN	the sum of ADP, AMP and ATP
ADP	adenosine-5'-diphosphate
ANT	adenine nucleotide translocator
ATP	adenosine-5'-triphosphate
ATP9	subunit 9 of the F ₀ -ATPase
bp	base pairs (of DNA)
BSA	bovine serum albumin
C	cytidine nucleotide
C ₄	four carbons
CAM	Crassulacean Acid Metabolism
CaMV	cauliflower mosaic virus
CAT	chloramphenicol acetyltransferase
cDNA	complimentary (or copy) DNA
Ci	Curie
CoA	Coenzyme A
CoQ	Coenzyme Q
COX1/2/3	mitochondrial genes encoding subunits 1, 2 and 3 of the cytochrome oxidase complex (complex IV)
COXI/II/III	cytochrome oxidase subunits I, II and III
CYC1	cytochrome c gene
Da	dalton
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
ddATP	2', 3'-dideoxyadenosine-5'-triphosphate
ddCTP	2', 3'-dideoxycytidine-5'-triphosphate
ddNTP	dideoxynucleotide-5'-triphosphates (N=A,C,G or T)
DEF A	<i>Antirrhinum</i> transcription factor
dGTP	2'-deoxyguanosine-5'-triphosphate
DHFR	dihydrofolate reductase
dicot	dicotyledonous plant
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide-5'-triphosphates (N=A,C,G or T)
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid (disodium salt)

EtBr	ethidium bromide
G	guanosine nucleotide
γ	gamma
g/l	grams per litre
g/ml	grams per millilitre
GAP	Wisconsin computer program for aligning protein and nucleotide sequences
GTP	guanosine -5'-triphosphate
GUS	β -glucuronidase
HAP1/2/3/4	yeast transcription factors
HeLa	human cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium salt
IPTG	isopropyl β -D-thiogalactoside
kb	kilobase pairs (of DNA)
kDa	kilodalton
Ki	inhibition constant
LB	Luria broth
λ gt10	an insertion vector for the construction of cDNA and genomic libraries
MCS	multiple cloning site
MES	2-[N-morpholino]ethanesulphonic acid
METC	mitochondrial electron transport chain
mg/ml	milligrams per millilitre
mjoules/cm ²	millijoules per square centimetre
monocot	monocotyledonous plant
MOPS	3-(N-morpholino)propanesulphonic acid
Mr	relative molecular weight
mRNA	messenger RNA
Mt1/3/4	enhancer elements 5' of human nuclear and mitochondrial genes
mtDNA	mitochondrial DNA
N	nucleotide (A,C,G,T or U)
NAD ⁺	nicotinamide adenine dinucleotide
NADH	β -dihydronicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NCS	non-chromosomal stripe
NMR	nuclear magnetic resonance
NOS	nopaline synthase gene
NTPs	nucleotide-5'-triphosphates (N= A,C,G,T or U)
OAA	oxaloacetate

OD	optical density
OXBOX	enhancer element 5' of several genes encoding protein subunits involved in oxidative phosphorylation
OXPHOS	used to denote genes encoding protein subunits involved in oxidative phosphorylation
PEP	phosphoenolpyruvate
PET494/111	yeast genes involved in the translational control of mitochondrial genes
pfu	plaque forming units
PIPES	1,4-piperazinebis(ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
poly dIdC	homopolymer polydeoxyinosinic-deoxycytidylic acid
polyA+	polyadenylated RNA
R or Pu	purine
RI	rotenone-insensitive
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RUBISCO	ribulose 1,5-bisphosphate carboxylase/oxygenase
S	Svedberg units
SDS	sodium dodecyl sulphate
T	thymidine nucleotide
TBE	Tris borate electrophoresis buffer
TCA	Tricarboxylic Acid Cycle
TE	10mM Tris-HCl, 1mM EDTA
T _m	melting temperature
TrisHCl	Tris-hydroxymethylaminomethane to pH with HCl
tRNA	transfer RNA
U	uridine nucleotide
UAS	upstream activator sequence
UTP	uridine-5'-triphosphate
UTR	untranslated region
UV	ultra-violet
v/v	volume per volume (as a percentage)
w/v	weight per volume (as a percentage)
x g	average maximum relative gravitational force
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
Y or Py	pyrimidine

CHAPTER 1

INTRODUCTION

1.1 GENERAL OVERVIEW

This thesis is aimed towards understanding the molecular control of mitochondrial biogenesis during plant development, particularly the biogenesis of the adenine nucleotide translocator (ANT) and the associated oxidative phosphorylation pathway. Throughout growth and development plant cells have varying requirements for energy. The primary source of this energy is mitochondrial oxidative phosphorylation. How the synthesis of the components of oxidative phosphorylation are coordinated in response to developmental or environmental stimuli has not yet been elucidated.

This chapter will summarise the changes in mitochondrial function and cellular energy requirements during plant development. These changes are dependent upon the activity and biogenesis of the oxidative phosphorylation pathway. Evidence for regulation of oxidative phosphorylation will be discussed along with various control mechanisms which may be utilised in response to metabolic signals. Since proteins associated with oxidative phosphorylation are encoded in both the nuclear and mitochondrial genome some form of coordinated gene expression may be required during the biogenesis of this pathway. The possibility of communication between the mitochondrial and nuclear genome and mechanisms for coordinated gene expression will be discussed. Finally, regulatory elements identified in mammalian systems that are specifically involved in the expression of *ANT* genes will be reviewed.

1.2 MITOCHONDRIAL FUNCTION

In plant metabolism the biochemical role of the mitochondrion ranges from general 'housekeeping' reactions to specialised tissue specific functions. Many of these pathways compete for the same redox cofactors. In the case of the NAD(H) cofactor, oxidative phosphorylation associated enzymes reoxidise it for further participation in metabolic reactions. Hence, the capacity of the pathway may be vital during periods of high metabolic activity. This section introduces the various components of

oxidative phosphorylation along with metabolic pathways characteristic of plant mitochondria. It highlights the complex role of mitochondria during plant development and the close relationship between various pathways and the mitochondrial electron transport chain (METC).

1.2.1 Oxidative phosphorylation and the TCA cycle

Oxidative phosphorylation and the TCA cycle are important for the production of energy in the form of ATP or reducing equivalents and carbon skeletons for biosynthesis. Their biochemical roles can be described as 'housekeeping' since cell metabolism is dependent on their products and indeed, they are conserved between mitochondria from all the eukaryotic kingdoms (Douce, 1985; Moore and Rich, 1980). There are several reviews which describe the reactions in detail (Douce, 1985; Moore and Rich, 1985; Wiskich and Dry, 1985). The basic reactions are shown in figure 1.1.

1.2.1.1 Oxidative phosphorylation

Oxidative phosphorylation constitutes the reactions, involving many complex enzymes, which convert chemical potential energy into a form the plant can utilise i.e. ATP (Figure 1.1). These include the enzymes of the METC (Douce, 1985) and the ATP synthase (Vignais and Satre, 1984). Additionally, the ANT protein is closely associated with oxidative phosphorylation since it is required to transport the ATP from the mitochondrial matrix to the cytoplasm (Klingenberg, 1980; 1988).

In plant mitochondria there are at least five potential entry sites for electrons (derived from metabolite oxidation) into the cytochrome pathway which all independently reduce ubiquinone. The two usual entry sites are the rotenone-sensitive NADH dehydrogenase (complex I) and succinate CoQ reductase (complex II). Other entry sites are rotenone-insensitive (RI) NAD(P)H dehydrogenases which are not coupled to oxidative phosphorylation or the TCA cycle. A proton gradient is generated across the inner membrane by the METC which pumps protons out of the mitochondrial matrix. Its electrochemical energy is used to transport the adenine nucleotides across the inner membrane (Heldt *et al.*, 1972) and to phosphorylate ADP to ATP (Cross 1981). Consequently, the production of ATP is coupled to the cytochrome pathway via the proton

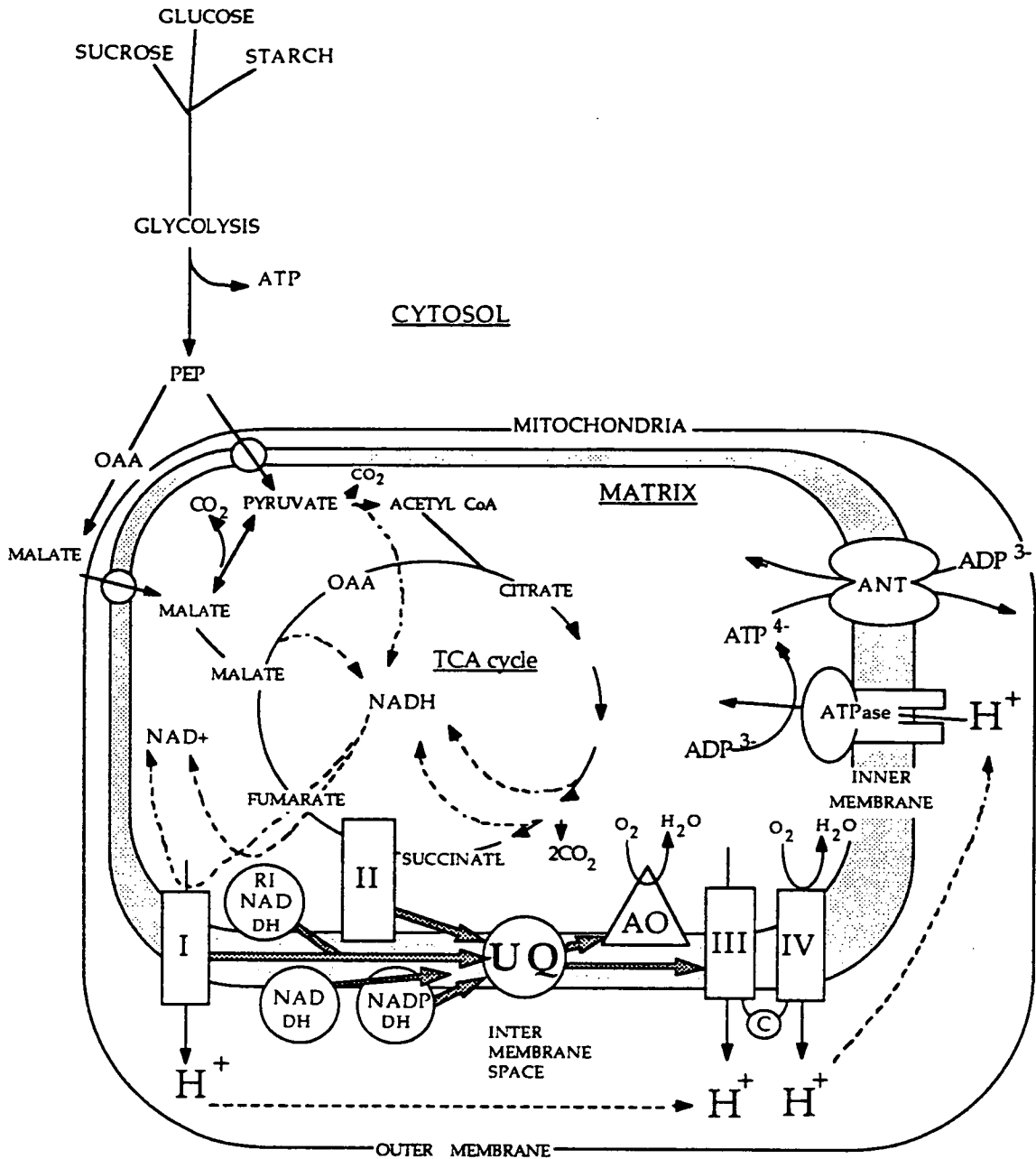


Figure 1.1

A summary of the interactions between the TCA cycle and the oxidative phosphorylation pathway during the oxidation of metabolic substrates.

A key of the abbreviations is below:

AO- alternative oxidase OAA- oxaloacetate

PEP- Phosphoenolpyruvate

I, II, III and IV- represent the complexes of the mitochondrial transport chain.

DH- dehydrogenases

ANT- adenine nucleotide translocator

gradient (Mitchell, 1980). *In vitro*, using isolated mitochondria, which are supplied with excess oxidisable substrate, the electron flux through the cytochrome pathway can be manipulated by either uncoupling the mitochondria (state 3) or by limiting the ADP supply for phosphorylation (state 4). State 3 represents maximum electron flux and state 4 represents the minimum electron flux (Lambers, 1990). *In vivo*, mitochondria appear to have a flux somewhere between the two extremes (Douce, 1985; Wiskich and Dry, 1985). This has been demonstrated in many different plant cell types by treating intact tissue with either an uncoupler (stimulates respiration) or oligomycin (inhibits respiration) (Lambers, 1990).

1.2.1.2 TCA cycle

DNA, RNA and protein synthesis all utilise carbon skeletons which are derived from the TCA cycle intermediates. Therefore, a cell must maintain the TCA cycle since the carbon skeletons are vital for basal metabolism. Both carbon input and oxidising equivalents are required for TCA cycle turnover.

The carbon input to the TCA cycle can be derived from carbohydrate via glycolysis in the form of pyruvate or C₄ acids (Douce, 1985; Wiskich and Dry, 1985; Davies, 1979). Plants, unlike animals, do not use fatty acid β -oxidation as a carbon input for the TCA cycle (Thomas and McNeil, 1976; ap Rees, 1990). Pyruvate is converted to Acetyl CoA in the mitochondrion by pyruvate dehydrogenase. C₄ acids, usually malate, are transported into the mitochondrion for oxidation or stored in the vacuole (Lance and Rustin, 1984; Bryce and ap Rees, 1985b). Malate can either replenish TCA cycle intermediates (the anaplerotic role) or be converted to pyruvate by malic enzyme (section 1.2.2.1).

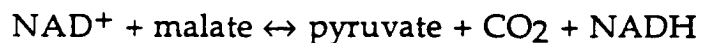
The oxidation of TCA cycle intermediates is coupled to the cytochrome pathway via complex I and complex II. Complex I oxidises NADH and is vital to maintain the TCA cycle turnover since NAD⁺ is required as an oxidising agent. Complex II tightly couples the TCA cycle to the cytochrome pathway since it is a TCA cycle dehydrogenase and is required for turnover. Both complex I and complex II reduce ubiquinone so the turnover of the TCA cycle is dependent on a constant flux of electrons through the cytochrome pathway.

1.2.2 Plant mitochondrial enzymes and their putative role in the maintenance of the TCA cycle

Due to the sessile and autotrophic nature of plants their cells must be tolerant of nutritional and environmental extremes. Consequently, rates of metabolism may vary over extremes; low rates of metabolism can result in both an inhibition of glycolysis (low carbon input into the TCA cycle) and a low rate of respiration (low cytochrome pathway flux) while high rates of biosynthesis may result in a depletion of TCA cycle intermediates or high levels of NADH in the mitochondrial matrix (NADH inhibits TCA cycle dehydrogenases (Pascal *et al.*, 1990)). These biochemical changes can inhibit the TCA cycle which may be detrimental to the plant if sustained over a long period. The enzymes discussed in this section are either unique to plants or more abundant than their animal equivalents. They all have a putative role in the maintenance of TCA cycle turnover.

1.2.2.1 NAD-malic enzyme

The biochemical role of NAD-malic enzyme in plants is poorly understood. Its ubiquitous nature in plants suggests a 'housekeeping' type role but it has also been recruited by some plants to perform specific CO₂ concentrating reactions (Douce and Neuburger, 1989; Wedding, 1989). The enzyme catalyses the reversible decarboxylation of malate in the mitochondrial matrix by the reaction below:



The carboxylation reaction has an anaplerotic function by maintaining C₄ acid input into the TCA cycle and may be important during intense biosynthetic activity. All plant cells have malic enzyme activity and its decarboxylation activity to maintain carbon input into the TCA cycle could be its major function in plant metabolism. Plant mitochondria do not oxidise cytosolic pyruvate efficiently, possibly as a consequence of a slow pyruvate transport mechanism from the cytoplasm to the matrix (Day and Wiskich, 1984). However, malate is readily oxidised by plant mitochondria (Douce and Neuburger, 1989) and may even be a major end product of glycolysis (Lance and Rustin, 1984). Additionally, malate oxidation in starved cells could be used as a temporary source of carbon to

maintain TCA cycle turnover. Plant cell vacuoles have a large store of malate and in extreme cases of starvation protein degradation may provide a source of C4 acids (Douce *et al.*, 1991). Likewise, during seed imbibition the role of malic enzyme may be critical in providing an immediate carbon input for oxidation and initial energy production in the absence of glycolysis (Bewley and Black, 1985).

In some C4 and CAM plants NAD malic enzyme has a specialised role in supplying CO₂ for photosynthetic reactions (Gardeström and Edwards, 1985). In C4 plants malate is decarboxylated in the bundle sheath cells. In CAM plants CO₂ is fixed during the night and malate is stored in the vacuole. Malate is subsequently exported to the mitochondrion during the day when photosynthetic reactions can assimilate the CO₂.

1.2.2.2 Alternative oxidase

The alternative oxidase is situated in the inner membrane of plant mitochondria and can oxidise the ubiquinone pool (Lance *et al.*, 1985; Hiser and McIntosh, 1990). It is a major component of the cyanide resistant pathway and its engagement is not energetically beneficial to the plant cell as it is non-phosphorylating. The role of this enzyme in metabolism is unique to plants since there are no corresponding enzymes in animal mitochondria. It has been implicated in several different metabolic functions including an overflow pathway when flux through the cytochrome pathway is saturated (De Visser *et al.*, 1986), oxidising excess carbohydrate (Lambers, 1985) and thermogenic reactions (Meuse 1975)(thermogenesis will be discussed in section 1.2.3).

Saturation of the cytochrome pathway has been simulated by treating *Neurospora* and cultured *Glycine max* cells with chloramphenicol to decrease the number of cytochrome pathway components (Lambowitz *et al.*, 1988; De Klerk-Kiebert *et al.*, 1982). Depletion of copper also reduces cytochrome pathway capacity by reducing cytochrome oxidase (Downie and Garland, 1973). In both examples smaller cytochrome pathway capacity and concomitant saturation of the pathway leads to the development of cyanide resistant respiration. In an unperturbed system such a stimulation of alternative oxidase activity may quickly generate ATP, via a complex I generated proton gradient, and might precede biogenesis of the cytochrome pathway (Lambers, 1985). Alternatively, it

may be to ensure TCA cycle turnover through maintenance of an oxidised ubiquinone pool.

1.2.2.3 Rotenone-insensitive dehydrogenase

Plant and animal mitochondria both have a rotenone-sensitive NADH dehydrogenase (complex I) in the inner membrane. Plant mitochondria also have three additional RI NAD(P)H dehydrogenases which are not coupled to oxidative phosphorylation. Two of these dehydrogenases oxidise NADH, one the cytosolic supply (RI external dehydrogenase) and the other the matrix supply (RI internal dehydrogenase). The third oxidises the cytosolic supply of NADPH. All three RI dehydrogenases reduce the ubiquinone pool and therefore can oxidise NAD(P)H in association with both the alternative oxidase and the cytochrome pathway.

The physiological function of these additional dehydrogenases is not known but some roles associated with the specialised functions of plant mitochondria have been speculated. Møller and Palmer (1982) determined that NADH will be preferentially oxidised by complex I rather than the RI internal dehydrogenase. If the matrix concentration of NADH increases, oxidation by the RI internal dehydrogenase will also be significant. This may occur when either NAD malic enzyme (section 1.2.2.1) or glycine decarboxylase (section 1.2.4) are actively producing NADH in addition to the TCA cycle dehydrogenases. NADH also increases if complex I is inhibited by adenylate control (section 1.4.1.1). Under these conditions, RI internal dehydrogenase may act as a bypass for complex I (Møller and Lin, 1986). Similarly, during adenylate control, alternative oxidase acts as a bypass for the cytochrome pathway. Thus, it is possible to envisage a non-phosphorylating pathway which bypasses the phosphorylating pathway to maintain TCA cycle turnover (Lance *et al.*, 1985; Palmer and Ward, 1985).

1.2.3 Thermogenic reactions

Certain animal adipose tissues (brown fat) contain specialised mitochondria which produce heat by uncoupling respiration from oxidative phosphorylation (Nicholls, 1984). These thermogenic mitochondria have a special protein, the uncoupling protein, which causes the inner membrane to become permeable to protons (Kozak *et al.*,

1988; Klingenberg, 1990). Plants are not known to have an equivalent uncoupling protein but in some specialised plants, such as the *Arum* species, mitochondria do have a thermogenic function (Laties, 1982; Meeuse, 1975) Heat is used to volatilise insect attractants during flowering and may increase the temperature in the flower by up to 15°C. The non-phosphorylating pathway, utilising RI internal dehydrogenase and alternative oxidase, has been postulated to have a function in uncoupling mitochondria. Proteins of the non-phosphorylating pathways are particularly prevalent in *Arum* thermogenic tissues since alternative oxidase capacity may be up to ten times greater than the cytochrome pathway (Moore and Rich, 1985; Elthon *et al.*, 1989). Energy is provided by rapid mobilisation of starch reserves via glycolysis (ap Rees *et al.*, 1977) and respiration. In fact, MacDougall and ap Rees (1991) have demonstrated that flux through the TCA cycle may increase by 25 fold.

An additional point of interest is that the thermogenic function of plant mitochondria may be more active at lower temperatures (Douce *et al.*, 1987). This may give plants added protection from frost damage and demonstrates their versatility in response to the changing environmental conditions.

1.2.4 Photorespiration

Photorespiration is an elaborate carbon salvage pathway which utilises enzymes in the chloroplast, mitochondria and peroxisome (Ogren, 1984) (Figure 1.2). Glycine decarboxylase and serine hydroxymethyltransferase are plant mitochondrial enzymes associated with photorespiration. These enzymes are induced in the mitochondria of cells which have a significant activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) (Rawsthorne *et al.*, 1988; Hylton *et al.*, 1988). Glycine decarboxylase produces large amounts of NADH in the mitochondrial matrix and has a low K_i (15mM) with respect to NADH. Consequently, NADH has to be rapidly reoxidised to prevent inhibition of glycine decarboxylase. It has been proposed that the NADH is reoxidised by the reversal of malate dehydrogenase and then transferred, by means of a malate/oxaloacetate shuttle, to the cytosol (Dry *et al.*, 1987). It is then utilised for the reduction of hydroxypyruvate to glycerate in the peroxisome.

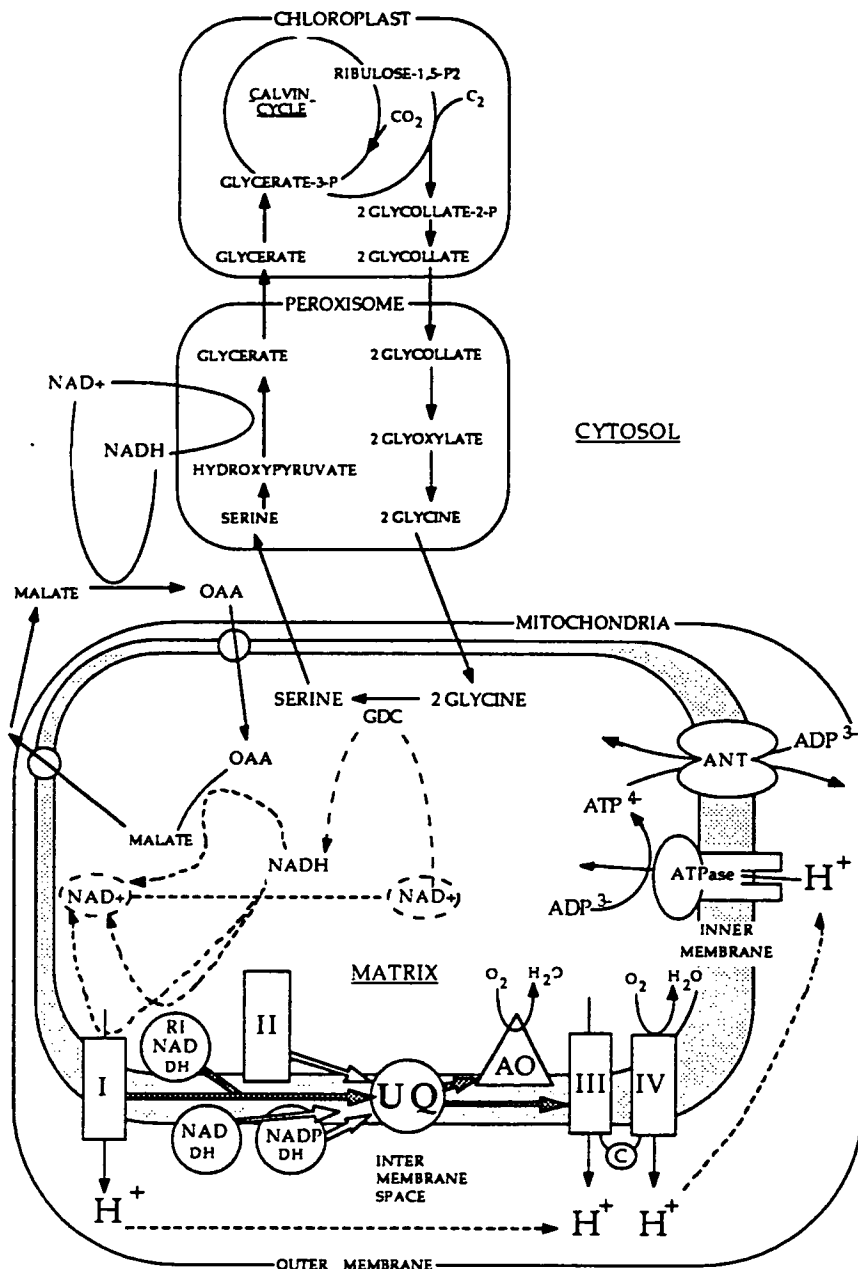


Figure 1.2

The interaction between the oxidative phosphorylation pathways and the photorespiration pathways.

A key of the abbreviations is below:

AO- alternative oxidase OAA- oxaloacetate

GDC- glycine decarboxylase

I, II, III and IV- represent the complexes of the mitochondrial transport chain.

DH- dehydrogenases

ANT- adenine nucleotide translocator

Photorespiration must compete with the TCA cycle for NAD⁺, access to the respiratory pathway, and malate dehydrogenase which catalyses the reverse reaction for each pathway. Wiskich *et al.* (1990) suggest that the two pathways may be physically separated in the matrix. This would explain the limited access of NADH generated by glycine decarboxylase to the respiration pathway (Day *et al.*, 1983) and how malate dehydrogenase can catalyse its forward and reverse reactions simultaneously.

1.2.5 Glyoxylate cycle

The glyoxylate cycle functions to convert lipids to sucrose which can be transported to other cells to support new growth or stored (Beever, 1980)(Figure 1.3). This cycle, which utilises enzymes in glyoxysomes, mitochondria and the cytosol, is especially important during germination in oil storing seeds and during senescence (Graham *et al.*, 1992). The mitochondrial function in this pathway is to convert succinate, formed by isocitrate lyase in the glyoxysome, to oxaloacetate. Oxaloacetate then leaves the mitochondria, is converted to phosphoenolpyruvate (PEP) and subsequently, to sucrose by way of gluconeogenesis. TCA cycle dehydrogenases are used for these reactions and the flux through the pathway generates reducing equivalents which are reoxidised by the METC for ATP production. There is a characteristic rise in respiration rate associated with germination and senescence which correlates with the increased amount of NADH generated by the glyoxylate cycle (Tetley and Thimann, 1974).

1.2.6 Summary

Some of the unique metabolic activities of mitochondria during plant development have been discussed above. It is obvious that in addition to housekeeping functions, which are vital to each cell, several specialised pathways are also dependent on mitochondrial enzymes. To ensure that the correct complement of enzymes are present at each developmental stage, mitochondrial biogenesis continues throughout development. This is clearly demonstrated by the tissue specificity of glycine decarboxylase (Rawsthorne *et al.*, 1988; Hylton *et al.*, 1988) and alternative oxidase (Elthon *et al.*, 1989). Changes in the expression of mitochondrial encoded polypeptides have been found to be affected both by environmental

factors, such as heat shock (Nebiolo and White, 1985) and anoxia (Couée *et al.*, 1992), and by developmental factors (Newton and Walbot, 1985).

The biochemical differences which are observed at various stages of development may account for the varied mitochondrial morphology that is seen between plant tissues (Douce, 1985). Occasionally subpopulations of mitochondria have been isolated from one tissue (Couée *et al.*, 1992). Whether these subpopulations represent different biochemical functions within one cell type is not known. As several reactions may directly compete for the same matrix cofactors, mitochondrial subpopulations may be a plausible mechanism to separate the different mitochondrial functions. However, Wiskich *et al.* (1990) could find no evidence of mitochondrial subpopulations to account for the different, photorespiration and TCA cycle related, malate dehydrogenase pools in leaf mitochondria. They concluded that the matrix is probably subdivided into distinct domains to account for the different activities.

All mitochondrial functions are closely related to respiration rate since reducing equivalents must be reoxidised to maintain turnover of the various pathways. A combination of non-phosphorylating and phosphorylating respiration ensures that metabolism is not inhibited even if energy is wasted. How the capacity for respiration and oxidative phosphorylation change with development and how these changes are regulated during sudden alterations in metabolic activity will be discussed further in the next section.

1.3 RESPIRATORY ACTIVITY AND CAPACITY IN PLANT TISSUES

Plant respiration, in combination with oxidative phosphorylation, is coordinated to meet the energy demands of the cell. Additionally, non-phosphorylating respiration may be used to reoxidise excess reducing equivalents when energy levels are high. Both total respiration and the contribution of phosphorylating and non-phosphorylating oxidation have been shown to vary between developmental stages and with environmental stimuli. This section will describe the variation that has been observed.

1.3.1 Developmentally induced changes in respiration of intact tissue

1.3.1.1 Germination.

During the early stages of seed imbibition and germination, respiration rates increase rapidly and are related to an increase in mitochondrial enzyme activities (Simon, 1984; Bewley and Black, 1985). After seed hydration, respiratory activity can increase either by activation of pre-existing mitochondrial enzymes or by *de novo* protein synthesis and subsequent mitochondrial biogenesis. *Pisum sativum* has been shown to utilise pre-existing mitochondria since their initial respiration burst is not perturbed by cycloheximide, an inhibitor of cytoplasmic protein synthesis (Morohashi, 1980). Additionally, recent work on maize embryos and sunflower seeds indicates that pre-existing mitochondria are preserved in quiescent embryos since both cytochrome oxidase and ATPase activities can be measured before imbibition (Ehrenshaft and Brambl, 1990; Attucci *et al.*, 1991). In contrast, experimental evidence from work on peanut showed that initial respiration in this species is inhibited by cycloheximide (Morohashi *et al.*, 1981). This suggests that *de novo* protein synthesis is vital for early mitochondrial function. Likewise, respiration increases in maize embryos which follow the initial burst, seem to coincide with active mitochondrial biogenesis (Ehrenshaft and Brambl, 1990). In summary, the mitochondrion is vital for germination in all seeds but current data indicates the importance of mitochondrial biogenesis to generate energy early after imbibition, is varied.

1.3.1.2 Roots

Roots have been shown to have their highest respiration rates in the developmentally younger root tip region (Yemm, 1965). Cells between the quiescent centre and elongation zone are undergoing active division and differentiation into cortical and stelar tissue. This presumably requires considerable energy and carbon intermediates from the mitochondria for biosynthesis and, consequently, mitochondrial biogenesis is also prevalent in this tissue (Kuroiwa, 1992).

1.3.1.3 Leaves

In photosynthetic tissue, respiration is most easily measured in the dark since light respiration is a combination of photorespiration and mitochondrial respiration. Dark respiration is primarily mitochondrial. Azcón-Bieto *et al.* (1983b) have measured dark respiration of maturing leaves from *Phaseolus vulgaris* and found that respiration in mature leaves is 3-fold less than in younger leaves. As determined by measuring respiration in the presence of an uncoupler this decrease seems to correlate with a decrease in capacity of the cytochrome pathway. The capacity of the cyanide resistant alternative oxidase remains constant during maturation. A similar observation was made by Owen *et al.* (1986) who isolated protoplasts from *Hordeum ditichum* (barley) leaf sections. Respiration per protoplast decreased up the leaf blade as the cells matured.

1.3.1.4 Anthers

Mitochondrial biogenesis has been reported to occur during early anther development in two specific cell types (Lee and Warmke, 1979). In sporogenous tissue, which gives rise to pollen mother cells, increases in mitochondria by up to 20 fold were reported and in tapetal cells mitochondria increase by up to 40 fold. It is postulated that the increase in mitochondria occurs in response to a large demand for energy during pollen development (Warmke and Lee, 1978). Energy is required by the tapetal cells for biosynthesis to support pollen cell development, while meiotic cell division itself may also require energy. Currently the precise molecular and biochemical events underlying pollen development are not well characterised.

Most cytoplasmic male sterile plants have rearrangements in the mitochondrial genome which are associated with abortion of young microspores. A possible result of these changes may be to prevent mitochondria from supplying enough energy for pollen development to proceed normally (Warmke and Lee, 1978). Since the number of mitochondria in both sporogenous and tapetal cells are identical in fertile and sterile maize plants (Lee and Warmke, 1979) mitochondrial function must be perturbed. This observation suggests that mitochondrial number may be determined genetically prior to an increased demand for energy.

Extensive research into mitochondrial biogenesis during pollen development is needed to elucidate the mechanisms which regulate the observed increases in mitochondria in tapetal and sporogenous tissue.

1.3.1.5 Storage tissue

Potato tuber respiration is reduced to a very low basal level when tubers are detached from the parent plant (Dizengremel, 1985). This represents the onset of dormancy. However, mitochondria in the tuber seem to remain intact since they are readily extracted (Dizengremel, 1985). A dramatic increase in respiration can be induced by treating the dormant tuber with ethylene (Hemberg, 1985) or by aging tuber slices (Laties, 1980). These changes are associated both with activation of pre-existing mitochondria and with mitochondrial biogenesis.

1.3.1.6 Senescence

The senescence of plant tissue is associated with a decrease in RNA and protein synthesis along with a characteristic rise in respiration (Noodén, 1988). Most other cellular processes decline during this period. Although senescence appears to be a general shut down of metabolic activities, the process is highly coordinated since RNA and protein synthesis are necessary (Yu and Kao, 1981). Since respiratory inhibitors have been shown to prevent the onset of senescence, the process is also energy dependent (Tetley and Thimann, 1974; Satler and Thimann, 1983). In fact, mitochondria are maintained longer than other organelles (Noodén, 1988; Thomas and Stoddart, 1980) indicating the importance of an energy supply.

A similar increase in respiration is observed during fruit ripening. This increase has been termed the 'respiration climateric' by Kidd and West (1925). While ripening of fruit is inhibited by treatment with cycloheximide the respiration climateric is not inhibited (McGlasson *et al.*, 1971). This suggests that the increase in respiration represents an increase in the activity of pre-existing mitochondria rather than active mitochondrial biogenesis.

1.3.2 Environmentally induced changes in respiration of intact tissue

1.3.2.1 Substrate availability

As plants are autotrophic their major substrates for respiration and energy production are sucrose and starch which are products of photosynthesis (ap Rees, 1990). Sucrose made in photosynthetic tissues is distributed to non-photosynthetic tissues via the phloem. During normal photosynthesis, sucrose is in excess with the result that starch reserves in all cells are replenished (ap Rees, 1987). Depletion of these reserves in the dark or during drought, when photosynthesis is inhibited due to closed stomata, can lead to a reduction in available substrates (Douce *et al.*, 1991). Fluctuations in substrate availability have been shown to affect respiration.

1.3.2.1.1 Photosynthetic tissue

The importance of mitochondria and respiration in leaves is more complicated than in non-photosynthetic tissue as there are two potential sources of ATP, oxidative phosphorylation and photophosphorylation. Heber (1974) postulated that photosynthetic cells would have a high concentration of cytosolic ATP in the light. This ATP, produced by photophosphorylation, would be sufficient to supply the energy requirements of the cell and be high enough to inhibit mitochondrial oxidative phosphorylation. This is termed photoinhibition. The rate of mitochondrial respiration in photosynthetic tissue was therefore expected to have an inverse correlation with the rate of photosynthesis. However, several independent researchers have since shown that ATP concentration is higher in the cytosol the dark than in the light (Stitt *et al.*, 1982; Bown and Nicholls, 1985), which contradicts the hypothesis of Heber (1974). Mitochondrial oxidative phosphorylation has since been shown to be essential for supplying the cytosol with ATP during photosynthesis (Kromer *et al.*, 1988). In addition, maize mitochondrial mutants, called nonchromosomal stripe (NCS), have a phenotype with defective chloroplasts (Roussel *et al.*, 1991). The mutations are in the mitochondrial genes encoding subunits of the METC. This suggests that mitochondrial function is important for the correct development and function of chloroplasts.

A number of observations are consistent with the suggestion that oxidative phosphorylation proceeds during photosynthesis. Graham *et al.* (1980) have shown that TCA cycle reactions operate during photosynthesis. Also, glycine decarboxylase produces large amounts of NADH in the matrix associated with photorespiration (Wiskich *et al.*, 1990). NADH must be reoxidised efficiently to avoid inhibition of glycine decarboxylase. Both these pathways utilise the METC to reoxidise reducing equivalents that have accumulated (sections 1.2.2.2 and 1.2.4).

1.3.2.1.2 Non-photosynthetic tissue

Non-photosynthetic tissues generally act as sinks for substrates and are dependent on sources, such as photosynthetic tissue and storage organs. Respiration changes, in response to substrate levels, have been well documented. They can be split into two categories, short and long term responses. Short term changes in respiration are discussed in section 1.4.1.2.

Several model systems are used to investigate long term changes of substrate levels in roots. Such changes are achieved by either excising roots, to separate them from the source (Brouquisse *et al.*, 1991), darkening the shoot (Farrar, 1981) or pruning selectively (Bingham and Farrar, 1988). Pruning leaves results in a decrease in transport of sucrose to the roots while pruning lateral roots increases sucrose transport to the seminal root. A decrease in substrate import consistently caused a decrease in respiration and a depletion of carbohydrate reserves. This is initially due to reduced activity of the cytochrome pathway. In the barley root system, changes in respiration due to an extended decrease or increase in substrate availability can be correlated with changes in the cytochrome capacity (Williams and Farrar, 1990; Bingham and Farrar, 1988). This is also true for maize roots (Brouquisse *et al.*, 1991) although the extent of the decrease, or the rate of decrease, in cytochrome capacity is much greater for barley. Cytochrome capacity in this context reflects the number of respiratory cytochrome complexes per mitochondria. Changes in the cytochrome capacity correlate with either an increase or decrease in the number of cytochrome complexes per mitochondrion.

A study of sucrose starved sycamore suspension cells by Journet *et al.* (1986) also showed a decrease in the mitochondrial cytochrome capacity. In this case the reduction of capacity was correlated with a reduction in the number of mitochondria per cell. It is not known if a similar response to starvation occurs in intact tissue.

1.3.2.2 Light and plant growth regulators

In barley leaf protoplasts, prepared from meristematic cells, respiration is modulated by both light and plant growth regulators (Owen *et al.*, 1987b). Light can act through either phytochrome or a blue light receptor to increase respiration. Gibberellic acid and abscisic acid were found to stimulate and inhibit respiration respectively. Additionally, the abscisic acid response was calcium dependent (Owen *et al.*, 1987a). These responses were absent in protoplasts isolated from more mature leaf cells.

The effect of these signals on mitochondria is not known. Robinson and Wellburn (1981) have demonstrated that isolated mitochondria are not sensitive to any of the stimuli including increased calcium levels. Yet calcium, calmodulin and other unknown regulatory proteins have been implicated in the *in vivo* responses (Wellburn and Owen, 1991). This suggests that a signal transduction pathway is involved in the response but the final regulatory points have not been identified. Possible targets are enzymes in the glycolytic pathway or mitochondrial pyruvate dehydrogenase both of which can limit carbon entry to the TCA cycle. Alternatively, the signals may affect mitochondria indirectly by regulating the energy consuming processes (This is analogous to the control of nitrate reductase by light, section 1.4.2.1.).

1.3.2.3 Salt induced respiration

The addition of KCl, NaCl or KNO₃ to excised tissue kept in distilled water increases respiration (Lambers, 1985). This phenomenon in plant tissues is known as salt respiration and is due to an active accumulation of salt thus avoiding dehydration (Jolivet *et al.*, 1990). This process requires ATP which is evident from the stoichiometry between net ion uptake and increased respiration (Farrar and Williams, 1991). Increased respiration seems to be due to an increase in flux through either the cytochrome

pathway or the alternative oxidase depending on the plant. A useful model system is nitrate uptake since this ion is also a plant nutrient. The addition of nitrate to *Pisum sativum* and *Plantago major* roots which have been nitrate starved stimulated respiration by inducing engagement of the alternative oxidase (De Visser *et al.*, 1986; Lambers *et al.*, 1981). In both these plants the cytochrome pathway was operating at full capacity. Engagement of the alternative oxidase is transient in each case and its decline may correlate with an increase of cytochrome capacity. Similar experiments with roots of barley by Bingham and Farrar (1989) indicated that the rise in respiration is due to an increase in flux through the cytochrome pathway with no engagement of the alternative oxidase. In this case there was no associated increase in cytochrome capacity since the cytochrome path was operating at less than 57% engagement prior to nitrate addition.

In some plants cytochrome capacity seems to fluctuate with demand for energy while others can maintain a low engagement without an appreciable loss of capacity. Therefore, it is clear that stimuli that cause mitochondrial biogenesis in some plants do not necessarily have the same effect in another system. The possible mechanisms that might be employed to regulate these different responses will be discussed in section 1.4.

1.3.2.4 Miscellaneous

Increases in respiration have also been observed in response to wounding, pathogen infection (Uritani and Asahi, 1980) and petal movements (Lambers, 1985). Wounding and infection are associated with very similar biochemical changes including the production of secondary products such as phenylpropanoids and phytoalexins (Uritani and Asahi, 1980). Energy required for the production of these products demands an increase in respiration and glycolysis. Likewise the increased energy demand for petal movement causes a rise in respiration.

The prolonged wound response and associated rise in respiration seems to be mediated by mitochondrial biogenesis as seen in potato tuber aging (section 1.3.1.6). For plant movement, increases in respiration are transient and correlate with the circadian rhythm of petal movement. The speed of this response suggests that pre-existing mitochondria are

utilised and that flux through the cytochrome pathway is reduced once the petals have opened or closed.

1.3.3 SUMMARY

This section has reviewed the developmental and environmental factors which affect respiration rates. The mechanisms employed to instigate changes in respiration have also been discussed. It is evident that mitochondrial respiration changes rapidly in plant tissue and is coordinated with energy requirements. Changes are mediated either by modifying activity of the relevant pathways or by varying their capacity. The nature of the regulatory signals relayed to mitochondria and the way these signals may be interpreted are discussed in the following section.

1.4 The control of respiration and oxidative phosphorylation

Mechanisms which control mitochondrial function and biogenesis are poorly understood. This section will address possible ways in which fine (immediate response) and coarse (slow response) controls regulate plant mitochondrial function and biogenesis.

1.4.1 Fine control

1.4.1.1 Respiratory control by adenine nucleotides

Generally the rate of oxidative phosphorylation is coordinated with the cells energy requirements. This metabolic *status quo* can be demonstrated by perturbing the system with specific inhibitors. Lowering the rate of protein synthesis with cycloheximide also lowers the rate of respiration (Cocucci and Marré, 1973). Similarly, lowering the rate of respiration with cyanide or a reduction in oxygen will lower the metabolic rate (Raymond *et al.*, 1987). Adenylate levels in a cell are thought to be responsible for much of this coordination between metabolic rate and oxidative phosphorylation although the proteins which exert the control are disputed (Dry *et al.*, 1987).

1.4.1.1.1 Mitochondrial proteins which may exert significant respiratory control via adenylate levels

The evidence for respiratory control is the fact that respiration in intact tissues can be stimulated by mitochondrial uncouplers which increase the permeability of the inner mitochondrial membrane to protons (Douce, 1985). This implies that respiration is restricted by the dissipation rate of the proton gradient. During state 4 respiration, when ADP is unavailable, the membrane permeability to protons controls the rate of respiration (Douce, 1985). Otherwise control is exerted either by the rate of ATP synthesis or by the ANT and phosphate carrier, which both supply substrate to ATP synthase. The exact mechanism of respiratory control by adenylates is unresolved. Chance and Williams (1955) first postulated a respiratory control mechanism which was a function of ADP availability. Subsequent theories have postulated that phosphorylation potential ($[ATP]/[ADP] + [Pi]$) (Klingenberg, 1961) or ATP/ADP ratio (Küster *et al.*, 1973) are more significant. The former is dependent on the thermodynamics of ATP synthesis while the latter is dependent on kinetics of the ANT.

External phosphate concentration has been reported to restrict respiration *in vitro* (Holian, 1977). This is unlikely to occur *in vivo* since the cytosolic phosphate concentration is normally much higher than the K_m value for the phosphate carrier (Rebeille *et al.*, 1984). Even starving cells of phosphate which reduces the cytosolic phosphate concentration by up to 10 fold has no obvious effect on respiration (Rebeille *et al.*, 1984).

The ANT is driven electrogenically by the membrane potential importing ADP^{3-} and exporting ATP^{4-} (Klingenberg, 1980). Consequently, a high ATP/ADP ratio is maintained in the cytosol at equilibrium. Although *in vitro* assays have shown that mitochondrial respiration is restricted when the external ratio of ATP/ADP exceeds 20 (Dry and Wiskich, 1982), at lower concentrations of AdN respiration is dependent on ADP concentration rather than ATP/ADP ratio (Jacobus, 1982). This suggests that the rate of ADP delivery to the mitochondria is more relevant for the control of respiration *in vivo*.

In vitro control analysis implies that the ATP synthase and the ANT are potentially very important in the regulation of respiration. In rat liver mitochondria control was found to be distributed between many of the steps from NADH reoxidation to the formation of ATP (Tager *et al.*, 1983; Groen *et al.*, 1982). Control coefficients were not fixed and were shown to vary depending on the rate of respiration and the supply of ADP. The highest control coefficient for the ANT was 0.3 which suggests a 2 fold increase or decrease in the protein could alter the rate of respiration by up to 15-20%. The ATP synthase may also have a similar control coefficient (Moreno-Sánchez, 1985). However, Padovan *et al.* (1989) have shown that the control of respiration in turnip mitochondria is primarily through the METC and that control contributed by ATP synthase and the ANT is negligible. Conversely, Hill (1990) has shown that in germinating cucumber cotyledons the ANT may contribute to control of respiration at specific stages of development. Padovan *et al.* (1989) argue that turnip mitochondria have more ANT per mg of mitochondrial protein than rat liver mitochondria. This and other differences in protein composition between plant and animal inner mitochondrial membranes may explain differences between the animal and plant data. However, since the mitochondrial environment *in vivo* is not well characterised, the *in vitro* experiments do not necessarily represent the physiological situation.

1.4.1.1.2 The *in vivo* environment with respect to adenine nucleotides

In order to determine whether control coefficients measured *in vitro* are physiologically significant, concerted efforts have been made to measure *in vivo* concentrations of adenine nucleotides. These data obtained from biochemical methods indicate that ATP/ADP ratios are not high enough to exert significant control via the ANT (Stitt *et al.*, 1982; Göller *et al.*, 1982). A problem with this conclusion is that measurements of nucleotide concentrations may be subject to large errors. Perchloric acid used to stop cellular metabolism may cause ATP hydrolysis. Also the short half life of ATP (about 8 seconds) means that cellular metabolism is not stopped fast enough to prevent significant turnover of ATP (Dry *et al.*, 1987). In addition, these techniques can not determine how much AdN is actually available i.e. how much is protein bound or free. Up to 40% of ATP in chloroplasts is estimated to be bound to protein (Inoue, 1978).

NMR methods have also been applied to the problem. This technique has the advantage of measuring nucleotide concentrations in living tissues and it can distinguish between protein bound and free AdN. According to Ackerman (1980) free adenine nucleotide is not as abundant as the original biochemical analyses suggested. Thus, ADP availability may be important for the control of respiration as postulated by Jacobus (1982). However, NMR data published by Roberts *et al.*, (1985) indicates that in maize roots the ATP/ADP ratio could be as high as 25. In this system, therefore, the ATP/ADP ratio may have significant control over respiration, via the ANT. Until the *in vivo* environment has been more fully described it will be hard to predict the true controlling factors of respiration.

1.4.1.2 Substrate control

Availability of substrate is a factor that can regulate respiration in the short term. Fluctuations in carbohydrate supply or regulation of flux through the glycolytic pathway account for substrate control of respiration.

A depletion of carbohydrate has been described in leaves of *Triticum aestivum* at the end of a dark period (Azcón-Bieto *et al.*, 1983a). In this starved state respiration can be stimulated by the addition of sucrose but not by an uncoupler. Respiration is, therefore, controlled by substrate supply rather than by adenylate control. However, a decrease in substrate does not always cause substrate supply to predominate over adenylate control. Williams and Farrar (1990) found that cytoplasmic carbohydrate decreases without an immediate effect on respiration rate. In this case, adenylate control determines the overall respiration rate.

Substrate supply can be limited by the adenylate control of glycolysis. This was demonstrated by Day *et al.* (1985a) who stimulated respiration by feeding *Lolium* leaves with non-glycolytic carbon sources such as glycine and malate. The addition of sucrose had no effect on the rate of respiration.

The fine control of respiration is dependent on mitochondrial protein composition and the cytosolic biochemical environment. These factors have a major role in determining which enzymes exert the control over the rate of respiration. They are variable between different plants (Day and

Lambers, 1983), depend upon the external environment and change as plant cells differentiate so it is hard to generalise about the control of respiration.

1.4.2 Coarse control

1.4.2.1 The regulation of mitochondrial biogenesis by substrate levels

As discussed above respiration may respond immediately to short term changes in substrate concentration but this is not always the case. However, there is a correlation between cellular carbohydrate levels and respiration over a long term period (Lambers, 1985; Williams and Farrar, 1990). Since these changes in respiration occur over several hours it is plausible that coarse control mechanisms are utilised. Certainly, as discussed above (section 1.3.2.2), several groups have evidence that mitochondrial capacity and number can change with long term changes in cellular carbohydrate levels (Journet *et al.*, 1986; Bingham and Farrar, 1988; Williams and Farrar, 1990; Brouquisse *et al.*, 1991). A prime candidate for the control signal is sucrose, or a similar sugar, although the precise mechanism is not obvious.

In bacteria glucose is known to activate and repress certain genes. Often the regulated genes are those involved in catabolism of other sugars or in utilisation of glucose itself. A well studied example is the lac operon (Watson *et al.*, 1987). Genes activated by this operon catabolise lactate but their expression is repressed in the presence of glucose. Such a simple substrate feedback control mechanism ensures that only the required enzymes are synthesised. This prevents the wasteful synthesis of unnecessary proteins. In yeast and *Drosophila*, glucose also regulates genes involved in its catabolism (Kapelli, 1986; Benkel and Hickey, 1987). Although these responses are well documented the exact mechanism of signal transduction is not known. To date, glucose has not been shown to interact with a receptor to instigate the regulatory cascade. As a result other glucose catabolites cannot be ruled out as having a regulatory role.

Glucose activates specific gene expression in the unicellular green algae *Chlorella*. Hilgarth *et al.* (1991) have shown that transcript levels for the glyceraldehyde-3-phosphate dehydrogenase gene and the *ANT* gene both increase by approximately 10 fold. This presumably reflects an increased

capacity to produce energy in order to sustain the high growth rate established when substrate is plentiful.

In plants, several genes involved in carbohydrate metabolism are regulated by sugars. However, the regulatory mechanism used to sense carbohydrate status is not known. The best documented is activation of sucrose synthase and invertase genes (Claussen *et al.*, 1986; Koch *et al.*, 1992; Masuda *et al.*, 1988). These responses are not confined to the addition of one particular sugar. Kaufman *et al.* (1973) demonstrated that sucrose, glucose or fructose will induce synthesis of invertase in *Avena* stem segments. Conversely, Sheen (1990) has demonstrated that the transcription of photosynthetic genes is suppressed by glucose.

High energy consuming reactions in plants may also be regulated by carbohydrate status. This is logical since cellular energy status is dependent on a supply of carbohydrate. A good example is nitrate reductase, the first enzyme in the nitrogen assimilation pathway, which requires reducing potential in the form of NADH. Initially this enzyme was thought to be regulated by light in a similar way to RUBISCO (Duke and Duke, 1984). Cheng *et al.* (1992) have demonstrated that nitrate reductase mRNA also accumulates in response to sucrose. It is now clear that light increases nitrate reductase indirectly by stimulating photosynthesis which in turn increases carbohydrate levels.

It is apparent that sugars are able to regulate some metabolic reactions in most organisms. Potentially the metabolic rate of a cell can be set by the available energy. Certainly there is a good correlation between the amount of available carbohydrate and the growth of a plant (Lamber, 1985; Farrar and Williams, 1991).

1.4.2.2 The regulation of mitochondrial biogenesis by metabolic activity

In addition to sugars, metabolic rate may be directly controlling respiration. Mitochondrial biogenesis is evident during the increased energy demands associated with wounding and pathogen infection (section 1.3.2.4). The alternative oxidase is particularly sensitive as discussed in section 1.2.2.2. A possible mechanism for this control has been tentatively proposed by Farrar and Williams (1991). They suggest that electron flux through the cytochrome pathway could be used as a

signal for the control of respiration. The metabolic rate and flux through the cytochrome pathway are closely linked by the respiratory control mediated by adenylate levels. Even in severely starved cells the respiration rate can be stimulated by uncouplers (Journet *et al.*, 1986; Brouquisse *et al.*, 1991). Therefore, the redox potential of the quinone pool would be a good indicator of the rate of ATP utilisation. In chloroplasts a mechanism is used to measure the redox state of the plastoquinone pool (Allen *et al.*, 1981). In the reduced state a protein kinase is activated which phosphorylates the light harvesting complex. Conversely, oxidised plastoquinone causes the dephosphorylation of the light harvesting complex. The function of this process is to correct any imbalance in the distribution of excitation energy between photosystem I and photosystem II. A similar mechanism might also be involved in the fine and coarse control of respiration.

Fine control may be mediated by phosphorylation of rate controlling enzymes to modify enzyme activity. There are at least two proteins subject to phosphorylation in mitochondria from etiolated oat shoots (Pike *et al.*, 1991). Pike *et al.* (1991) have identified the pyruvate dehydrogenase and the other is an unknown 67 KDa protein. Sommarin *et al.* (1990) have identified up to 5 different proteins which may be phosphorylated in potato tuber mitochondria. The inhibition of pyruvate dehydrogenase effectively blocks substrate entry to the TCA cycle. This will prevent TCA cycle turnover and reduce electron flux through the METC. Coarse control may be mediated by the phosphorylation of trans-acting regulatory proteins. In yeast genes encoding proteins of the METC have been shown to be coordinately regulated by the HAP1 trans-acting factor (Pfeifer *et al.*, 1987; section 1.5.1). A plant homologue for HAP1 has not been identified. If one exists its phosphorylation might change mitochondrial gene expression in a coordinate fashion. Certainly there are animal and plant trans-acting factors, SRF and DEF A, which are known to be phosphorylated (Prywes *et al.*, 1988; Schwarz-Sommer *et al.*, 1990). Additionally, post-translational modification of the SRF factors plays an important role in the regulation of gene expression.

1.4.2.3 The regulation of mitochondrial biogenesis by developmental factors

It is apparent that environmental stresses that cause an increase in ATP utilisation or a reduction in available respiratory substrates may cause a modification of mitochondrial function. It is not clear if this is achieved by changing the number or by changing the capacity of certain pathways in pre-existing mitochondria. A simple hypothesis is that mitochondrial biogenesis is regulated by the cells metabolic status. While such a mechanism is useful for modifying mitochondria in response to metabolic activity it does not exclude pre-programmed developmental control of mitochondrial number.

Generally metabolically active tissues, which are respiring at a high rate, have a larger number of mitochondria. In some instances the level cannot be regulated by metabolic status since mitochondrial number is set before the cell becomes metabolically active. Fruit ripening is such an example since the characteristic respiratory climateric is not inhibited by cycloheximide although other ripening responses are inhibited (Rhodes, 1980; McGlasson, 1971). This suggests that mitochondrial number in the fruit has already been set at a level which would be sufficient to account for the respiratory burst. Similarly, in tapetal cells the large increase in mitochondrial number may precede the increased demand for energy (Lee and Warmke, 1979). At present there is no evidence that plant mitochondrial genes respond to developmental signals.

1.4.3 Summary

It is clear that control of the METC and glycolysis by adenylates and substrate availability are important in controlling mitochondrial respiration. However, the relative contribution of a particular mechanism in any one tissue or plant is variable. The metabolic state of the cell appears to govern which control mechanism predominates in a tissue. Also, the relative concentrations of protein complexes from glycolysis to oxidative phosphorylation may play a role in predetermining where fine control is exerted. To fully comprehend the control of respiration it is important to understand how enzyme levels are monitored in the cell and

how these levels may vary in response to developmental or environmental stimuli (Figure 1.4).

As cells differentiate several different types of signal may be involved in the modulation of mitochondrial number and protein composition. There may be overriding developmental signals which can set levels according to the 'genetic plan'. These levels may be adjusted further according to the metabolic status of individual cells. To date, nothing is known about how the amount of individual mitochondrial proteins are regulated in plants. However, research on yeast and mammalian systems has identified some of the molecular elements of the regulatory process. The next section will discuss these recent discoveries.

1.5 Regulatory interactions between nuclear and mitochondrial genomes

Mitochondrial protein complexes involved in oxidative phosphorylation are comprised of proteins encoded in both the nuclear and mitochondrial genomes. This fact has led to the proposal that gene expression from the two genomes is coordinated to maintain the correct subunit stoichiometry. Additionally, genes encoding proteins involved in oxidative phosphorylation may be coordinately expressed as they are components of the same pathway. In this section I will discuss some of the research that has been done to investigate the coordination and regulation of biosynthesis of mitochondrial enzyme complexes.

1.5.1 Regulation of genes encoding yeast cytochrome oxidase

Two recent reviews describe the regulation and coordination of nuclear and mitochondrial gene expression in yeast (Forsburg and Guarente, 1989; Costanzo and Fox, 1990). Much of the research has centred on the yeast cytochrome c oxidase complex. Its three main subunits COXI, COXII and COXIII are mitochondrially encoded while the remaining 4 subunits are encoded in the nucleus. The regulation of genes encoding these subunits has been thoroughly dissected and is summarised in the following sections.

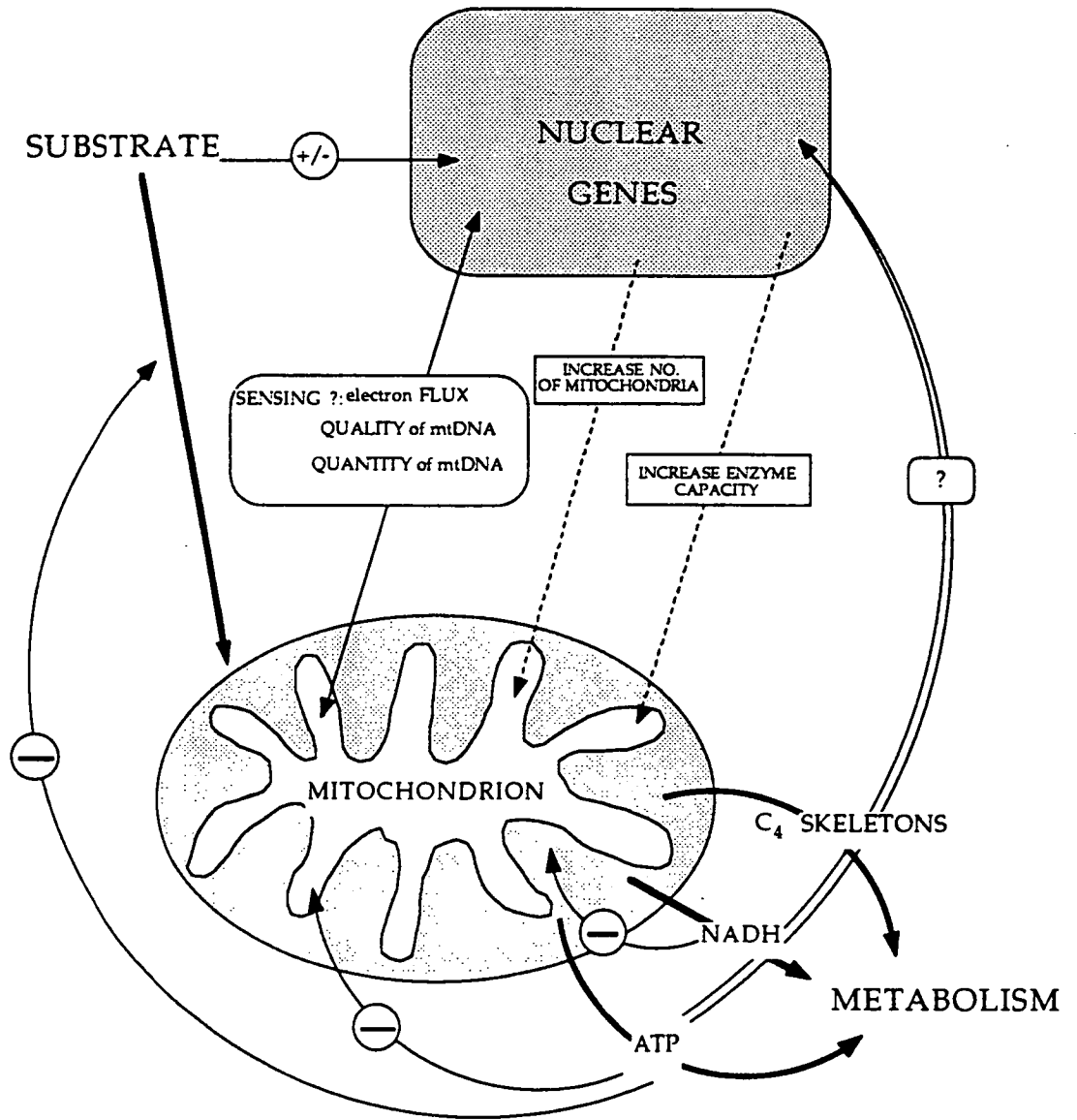


Figure 1.4

A summary of the potential regulatory factors discussed in this chapter which may affect the fine and coarse control of mitochondrial function during development.

1.5.1.1 Regulation of mitochondrial genes

Mitochondrial gene expression may be regulated at the level of transcription, processing or translation (Biswas, 1990; Mulligan *et al.*, 1991). Which of these predominates under different environmental conditions is not known. Certainly there is translational control of mitochondrial genes encoding cytochrome oxidase subunits during glucose repression and oxygen deprivation in yeast (Woodrow and Schatz, 1979). Genes which encode translational regulatory proteins are commonly mutated in the respiratory deficient *pet* strains. These include *PET111* and *PET494* which are both nuclear genes encoding mitochondrial proteins. They regulate *COX2* and *COX3* mRNA translation respectively. *PET494* is subject to transcriptional and translational control in response to carbon sources and oxygen levels (Marykwas and Fox, 1989). *PET494* represents a form of communication from nucleus to mitochondria. By modulating regulatory protein levels, such as *PET494*, the nucleus controls mitochondrial translation rate in response to the metabolic environment. In a similar manner to *PET494*, nuclear encoded genes for cytochrome oxidase are subject to transcriptional control in response to carbon sources and oxygen levels (Trueblood *et al.*, 1988; Forsburg and Guarente, 1989). Thus, it can be postulated that the coordinated synthesis of yeast cytochrome oxidase subunits encoded in nuclear and mitochondrial genomes is modulated by the metabolic environment of the cell.

1.5.1.2 Regulation of nuclear genes

Yeast activator proteins which regulate the transcription of nuclear encoded cytochrome oxidase genes are well characterised. These include haem responsive *HAP1* (Pfeifer *et al.*, 1987) and carbon source responsive *HAP2/3/4* multimeric complex (Olesen *et al.*, 1987; Olesen and Guarente, 1990). Additionally, the *HAP1* protein is indirectly sensitive to oxygen levels since several of the enzymes involved in haem biosynthesis are oxygenases (Forsburg and Guarente, 1989). In response to physiological stimuli the *HAP1* protein and *HAP2/3/4* complex bind specifically to upstream activator sequences (*UAS1* and *UAS2* respectively) to activate transcription (Guarente and Mason, 1983; Guarente *et al.*, 1984). As well as nuclearly encoded *COX* genes the *UAS* sequences are found upstream of other respiratory genes such as iso-1 cytochrome *c* (*CYC1*), iso-2

cytochrome c (CYC7) and cytochrome c1 (CYT1) (Forsburg and Guarente, 1989). The UAS sequences appear to have a role in the coordinated expression of the nuclear genes encoding respiratory proteins.

1.5.2 Coordination of oxidative phosphorylation related proteins

The conserved regulatory sequences UAS1/2 are located upstream of many yeast nuclear genes which encode proteins of the oxidative phosphorylation pathway (Trawick *et al.*, 1989; Winkler *et al.*, 1988). Several regulatory elements have been identified upstream of mammalian genes associated with oxidative phosphorylation (Evans and Scarpulla, 1989; Tomura *et al.*, 1990; Li *et al.*, 1990). First to be recognised was a regulatory element which bound a nuclear factor designated nuclear respiratory factor 1 (NRF-1). The core of the NRF-1 footprint has the sequence GCATGCGC. This element was identified upstream of the rat and human cytochrome c gene, the mouse mitochondrial RNA processing gene, the rat cytochrome c oxidase subunit VIc gene and the human cytochrome c1 gene (Evans and Scarpulla, 1989). Tomura *et al.* (1990) described a different regulatory element, consensus TAGAGACAAGGTT-TCACCA, which is upstream of the human genes encoding the ATP synthase β subunit, cytochrome c1 and the pyruvate dehydrogenase E1 α subunit. Finally, a third element was identified by Li *et al.* (1990) which was termed the OXBOX (consensus GGCTCTAAAGAGG). This element has been identified upstream of the human ATP synthase β subunit gene and the human *ANT-1* gene. The signals which act through these mammalian regulatory elements are presently unknown (section 1.6).

The yeast and mammalian regulatory elements discussed in this section are upstream of several nuclear genes involved in oxidative phosphorylation. Thus, they have the potential to coordinate expression of these genes. Further analysis of various mammalian regulatory elements should elucidate other factors involved in the regulation of expression. It will also reveal a role for each element in interpreting the cellular environment.

1.5.3 Coordination of nuclear and mitochondrial gene expression

If there is a coordinated production of yeast respiratory subunits encoded in the nuclear and mitochondrial genomes the UAS elements may also be

upstream of *PET494* and *PET111*. This would represent the simplest mechanism to coordinate expression of the two genomes. Recently it has been shown that neither HAP1 or the HAP2/3/4 complex bind upstream of *PET494* (Marykwas and Fox, 1989). Also, expression of *PET494* in response to oxygen is very different from nuclear genes encoding protein subunits involved in oxidative phosphorylation (nuclear OXPHOS genes) such as the *COX4/5/6/7* genes (section 1.5.1.2). *PET494* is regulated by oxygen at the translational level and is independent of haem (Marykwas and Fox, 1989). Other nuclear OXPHOS genes such as *ANT*, α and β subunits of ATP synthase are also subject to glucose repression (Szekely and Montgomery, 1984) although no UAS2 upstream elements have been identified. Thus, in yeast, *PET494* and genes encoding proteins involved in oxidative phosphorylation genes respond to the same signals, although, they are not necessarily conveyed by the same transduction pathway. The mechanism for the coordination of expression between the two genomes remains elusive.

A possible candidate for the first common regulatory protein of *PET494* and the OXPHOS genes is SNF1 which has been identified as a yeast protein kinase (Celenza and Carlson, 1986). Both *COX6* and *CYC1* require SNF1 to be released from glucose repression (Wright and Poyton, 1990). Wright and Poyton (1990) have postulated that SNF1 may act by affecting the synthesis of the HAP proteins or by affecting their function. Similarly SNF1 may act on other trans-acting factors which bind upstream of *PET494*. Certainly SNF1 is known to interact with other regulatory proteins such as SSN6 (Schultz and Carlson, 1987). How the specificity of the kinase is determined is currently unknown.

Some novel regulatory elements have been discovered upstream of the mammalian genes for cytochrome c1 and ubiquinone binding protein, subunits from complex III (Suzuki *et al.*, 1991). These elements Mt1, Mt3 and Mt4, have been shown to bind proteins from a HeLa cell nuclear extract. Mt3 and Mt4 are also present in the mitochondrial genome in the noncoding region containing the displacement loop (D Loop) and promoters. Potentially the presence of these elements in both mitochondrial and nuclear promoter regions would allow a single trans-acting factor to coordinate expression between the two genomes. Such a factor would be unique since it would have to be targeted to a nuclear and

mitochondrial location. Whether such a mechanism plays a role in the coordinated expression of the two genomes is not known.

Chomyn and Lai (1990) carried out experiments to determine if transcription is coordinated between the two genomes in bovine cells. Using probes for nuclear and mitochondrial genes for mammalian NADH dehydrogenase (complex I) they looked for coordinated changes in the level of transcripts. They determined that while the nuclear transcript levels changed between different bovine tissues the mitochondrial transcripts were maintained at a relatively constant level. This observation indicates that translational control may be more important in mammalian mitochondrial gene expression in an analogous way to the yeast system.

1.5.4 A mitochondrial signal?

An intriguing problem is whether information can be relayed from the mitochondria to the nucleus. Such a signal may be important to maintain the correct levels of mitochondria in a cell. Certainly the haem molecule, which originates from the mitochondria, is able to influence the expression of nuclear genes (Forsburg and Guarente, 1989). Also, quantity and quality of yeast mitochondrial DNA can modulate the expression of some mitochondria related nuclear genes (Parikh *et al.* 1987, 1989; Farrell *et al.*, 1990). Similarly, Lunardi and Attardi (1989) used a human cell line which lacks mitochondrial DNA to demonstrate an increase in the expression of the human *ANT2* gene. In other human cell cultures mitochondrial populations have been artificially depleted to about 10% of the normal level using ethidium bromide. In this case the mitochondrial levels are restored to the original level (King and Attardi, 1988). These observations imply that the nucleus can monitor levels of cellular mitochondrial DNA. If there are signals from the mitochondria to control their own replication and biogenesis, their nature is not obvious. Parikh *et al.* (1987) suggest two hypotheses for such a regulatory mechanism. First a DNA element in the mitochondrial DNA may act as a binding site for a nuclear 'sensor' protein. Alternatively, an unidentified peptide/protein may be synthesised by the mitochondria and exported to the nucleus. Similar mechanisms may be employed by plant cells to modulate the mitochondrial population in response to developmental cues.

1.5.5 Summary

A host of signals and regulatory elements have been implicated in the regulation of expression of nuclear and mitochondrial genes involved in oxidative phosphorylation. As yet, no mechanism has been demonstrated to coordinate the expression of the nuclear genes or coordinate the mitochondrial and nuclear genomes. In fact, coordinated expression between the nuclear and mitochondrial genome may not actually occur. Costanzo and Fox (1990) have postulated that the levels of the various respiratory complexes may be determined by a limiting subunit. Hence, different subunits may be limiting in different situations, such that no single regulatory system has overall control.

Similar problems of coordinated expression are being addressed for interactions between chloroplast and nuclear genomes. This thesis will not discuss the chloroplast data but a recent review summarises the progress to date (Taylor, 1989).

1.6 REGULATION OF SYNTHESIS OF THE ADENINE NUCLEOTIDE TRANSLOCATOR

Several regulatory elements have been described upstream of human *ANT* genes. It has been shown in humans that there are three *ANT* genes which are subject to differential or coordinate regulation (Lunardi and Attardi, 1991). Growth conditions, tissue specific factors and metabolic inhibitors have all been shown to affect expression. *ANT1*, which is expressed predominantly in muscle and heart tissue, is the only one to have an upstream OXBOX (Li *et al.*, 1990)(section 1.5.2). Thus, it can be hypothesised that the OXBOX element may direct muscle and heart specific expression although this has not been demonstrated experimentally. Specific mitochondrial regulatory elements, such as the OXBOX, are not the sole regulators of nuclear oxidative phosphorylation gene expression. Upstream of the human *ANT1* gene three distinct regulatory regions have been localised by transfecting a series of promoter-chloramphenicol acetyltransferase fusion constructs into myogenic cells (Li *et al.*, 1990). Each region was found to increase transcription by 2-3 fold although only one of these regions contains the OXBOX. Various combinations of these regions gives an additive increase in transcription,

thus, each region appears to have an independent activating function. Similarly, many different enhancer regions have been identified upstream of the ATP synthase β subunit gene (Tomura *et al.*, 1990) and the rat cytochrome c gene (Evans and Scarpulla, 1988, 1989). Binding sites for transcriptional activators such as ATF (Lin and Green, 1989) and Sp1 (Kadonaga *et al.*, 1986) have been identified in these regions. The presence of the Sp1 binding sites along with others, such as AP-2 and OCT-1 (Mitchell and Tjian, 1989), have also been noted upstream of the bovine and human *ANT* genes (Li *et al.*, 1989; Ku *et al.*, 1990; Powell *et al.*, 1989). Such binding sites are common upstream of nonrespiratory genes (Mitchell and Tjian, 1989). Dynan (1986) has suggested that Sp1 regulatory elements may be common in 'housekeeping' genes and which may explain the presence of Sp1 binding sites in the promoters and introns of oxidative phosphorylation genes. How all these different regulatory elements combine to give the observed *in vivo* expression patterns is presently unknown

1.7 SUMMARY

This section has discussed how changes in plant cell function depend on the control of mitochondrial activity and biogenesis, particularly the oxidative phosphorylation pathway which must be coordinated with the demand for energy and oxidation potential. Regulatory mechanisms employed by yeast and mammalian cells to control mitochondrial activity and biogenesis have been discussed. These range from fine to coarse control and include the identification of several regulatory elements with a putative role in the coordinate expression of mitochondrial proteins. As yet, similar regulatory mechanisms have not been identified in plant systems.

The more complex role of mitochondria in plant metabolism may require unique regulatory elements in addition to those that function in animal and yeast systems. We hope to identify these regulatory elements upstream of plant nuclear genes encoding mitochondrial proteins. Several nuclear genes encoding oxidative phosphorylation pathway proteins have been cloned from *Zea mays* for this purpose including two *ANT* genes (Bathgate *et al.*, 1989) and one ATP synthase β subunit gene (Winning *et al.*, 1990). The work described in this thesis will concentrate

on the two *ANT* genes since ANT protein levels may be important for fine control (section 1.4.1.1.1) as well as for regulation associated with changes in the capacity of the oxidative phosphorylation pathway. As described above for mammalian *ANT* (section 1.6), a combination of many different regulatory elements may be responsible for the expression pattern of *ANT* genes. I have attempted to identify regulatory elements in plant *ANT* genes with a view to establishing the molecular mechanisms involved in regulating plant mitochondrial biogenesis. The following chapters describe the approach taken and the results obtained.

CHAPTER 2

MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIAL

2.1.1 Plant material

Seed from *Zea mays* L., nuclear genotype B73, with the N (fertile) cytoplasm, was supplied by Pioneer Hi-bred International.

2.1.2 *Escherichia coli* strains and their genotypes

ED8654: *supE supF hsdR metB lacY gal trpR*

(Borck *et al.* 1976; Murray *et al.* 1977)

HB101: *supE 44 hsdS 20(r⁻ Bm⁻ B⁻) recA13 ara -14 proA 2 lacY 1 galK 2 rpsL 20 xyl -5 mtl -1*

(Boyer and Roulland-Dussoix 1969; Bolivar and Backman 1979)

JM101: *SupE thi Δ(lac proAB) F' [traD36 proAB+ lacI^q lacZ ΔM15]*

(Messing 1979)

JM109: *recA1 supE 44 endA1 hsdR17 gyrA 96 relA1 thi Δ(lac proAB) F' [traD36 proAB+ lacI^q lacZ ΔM15]*

(Yanisch-Perron *et al.* 1985)

2.1.3 Bacteriophage λ vector

λgt10: *λsr II° b 527 sr II3° imm 434 (sr I434+) sr II4° sr II5°*

(Huynh *et al.* 1985)

2.1.4 Bacterial plasmids

pUC19

Yanisch-Perron *et al.* (1985)

pK18

Pridmore (1987)

pGEM-1

Promega Corporation USA

pBS M13+

Short *et al.* (1988)

pBluescript II KS -

Short *et al.* (1988)

2.1.5 Code of practice

Cultivation of all genetically manipulated bacteria was carried out according to the code of practice of the local Genetic Manipulation Safety Committee.

2.1.6 *ANT* cDNA clones from maize

Three clones were isolated by Dr Alison Baker from a cDNA library constructed from 2.5-day-old dark-grown maize seedlings (maize line B37) in this laboratory. These clones were named pANT-1, pANT-2 and pANT-3. The pANT-1 clone is 1.2 kb and identical to pANT-3 except it is 49 bp longer at the 5' end (Baker and Leaver, 1985). The pANT-2 clone is shorter (size is undetermined) and encodes a different ANT protein to pANT-1. A fourth *ANT* cDNA was cloned from a cDNA library, constructed from 3-day-old dark-grown maize seedlings (MutinD-Fr7205034 hybrid seed) which was a gift from Dr K. Palme (Cologne), by Dr Brenda Winning in this laboratory. The clone was 1.6 kb, was identical to pANT-1 and was named pMANT1 (Winning *et al.*, 1991).

2.1.7 *ANT* genomic clones from maize

Two genomic clones were isolated by Dr Alison Baker from a library constructed from genomic DNA (maize line B37) digested with the restriction enzyme EcoRI. One clone designated *ANT1* corresponds to the pMANT1 cDNA while a second clone designated *ANT2* corresponds to the pANT-2 cDNA (Bathgate *et al.*, 1989). The cDNA and genomic clones are summarised in (Figure 3.1).

2.1.8 Gene nomenclature for the maize *ANT* genes

The gene nomenclature for the maize *ANT* genes has been confused by the variety of shorthand names which have been assigned to the two genes. The two maize *ANT* genes have been termed *G1* and *G2* (Baker, 1985), *ANT-G1* and *ANT-G2* (Bathgate *et al.*, 1989) or *MANT1* and *MANT2* (Winning *et al.*, 1992). None of these shorthand names fulfil the guidelines proposed by the 'Commission on Plant Gene Nomenclature' (Plant Mol. Biol. Rep. 10, 2-3, 1992). The names *G1/2* are unsuitable since generally the names of genes are expected to be mnemonic. In this case the abbreviation *ANT* is an appropriate name for these genes. The names

ANT-G1/2 are unsuitable since a hyphen should only be used to denote a mutant or allele. The names *MANT1/2* are unsuitable since the names of genera or species (in this case M for maize) should not be implied in shorthand gene names. Consequently, to comply with these guidelines the two maize ANT genes will be referred to throughout this thesis as *ANT1* and *ANT2*.

2.2 MISCELLANEOUS

2.2.1 Chemicals

All chemicals were purchased from Sigma Chemical Co. Ltd. or from BDH Chemicals Ltd. unless otherwise stated.

2.2.2 Enzymes

Restriction enzymes were purchased from GIBCO BRL Life Technologies Ltd and Amersham International Plc.

Reverse Transcriptase and the Riboprobe® gemini II kit were from Promega Ltd. The Multiprime DNA labelling system was from Amersham International Plc. Two sequencing kits were used, the T⁷Sequencing™ Kit and the Sequenase® Version 2.0 which were from Pharmacia LKB Biotechnology and United States Biochemical Corporation respectively.

2.2.3 Radiochemicals

α [³²P]dCTP (3000 Ci/mmol), γ [³²P]dATP (5000 Ci/mmol), α [³²P]UTP (800 Ci/mmol) and α [³⁵S]dATP (1000 Ci/mmol) were purchased from Amersham International Plc.

2.2.4 Photography

Photographs of agarose gels were taken using an HP4 camera with Type 667 positive/negative Polaroid film (Genetic Research Instrumentation Ltd.). Plant material was photographed with Kodacolor Gold 100ASA film.

2.2.5 Autoradiography film

The X-ray film used was Cronex-4® (Du Pont (U.K.) Ltd.) which was developed in an Agfa-Gaevart Gevomatic 60 automatic developer.

2.2.6 Centrifugation equipment

Beckman JA-14, JA-20 and JS-13.1 rotors were used in a Beckman J2-21 centrifuge. Bottles of 250 ml were used with the JA-14. Disposable 15 ml and 30 ml polypropylene tubes (Sarstedt Ltd.) or Corex® tubes (Fisons Scientific Equipment) were used in the JA-20 and JS-13.1 rotors.

Two Beckman ultracentrifuges a TL-100 and an L-70 were used with the TLA 100.3 and 70.1Ti rotors respectively. Beckman polyallomer bell-top Quick-Seal™ centrifuge tubes were used with these rotors for caesium chloride gradients for the isolation of DNA. Eppendorf™ 1.5 ml microcentrifuge tubes were used with an Anderman 5414 microcentrifuge for subsequent reactions and manipulations with isolated DNA.

2.3 STOCK BUFFERS AND MEDIUM

All solutions and reagents were stored at room temperature unless otherwise specified.

2.3.1 Bacterial and bacteriophage media

All strains of *Escherichia coli* were grown in Luria-Bertani medium (LB) or LB agar.

LB medium: 10 g/l Bacto tryptone (Difco Laboratories), 5 g/l Bacto yeast extract (Difco), sodium chloride 10 g/l, pH 7.2.

LB agar: As LB medium with the addition of 1.5% (w/v) Bacto agar (Difco).

Top agar: As LB medium with the addition of 0.7% (w/v) Bacto agar.

2.3.2 Antibiotics

Ampicillin: 50 mg/ml stock suspension in sterile H₂O from a Milli-Q® Plus water system (Millipore). Stored at -20°C.

Kanamycin: 10 mg/ml stock solution in sterile a Milli-Q® Plus water. Stored at -20°C.

2.3.3 Electrophoresis buffers

5xTBE: 445 mM TrisHCl, 445 mM boric acid, 10 mM EDTA pH 8.0.

10xMOPS: 0.2 M MOPS, 50mM NaOAc, 10 mM EDTA pH 7.0.
Stored at 4°C in a darkened bottle.

2.3.4 Transfer buffers

20xSSC: 3 M NaCl, 0.3 M Na citrate pH 7.0.

20xSSPE: 3.6 M NaCl, 0.2 M NaH₂PO₄.H₂O, 2 mM EDTA pH 7.7.

Depurinating: 0.25 N HCl

Denaturing: 0.5 M NaOH, 1.5 M NaCl.

Neutralising: 1.0 M TrisHCl, 2.0 M NaCl pH 7.0.

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 General conditions

All solutions, pipette tips and microcentrifuge tubes used in the isolation and manipulation of nucleic acids were autoclaved at 15 psi for 20 minutes before use. Milli-Q® Plus water was used at all times to prepare solutions. In addition glassware used for RNA preparations was baked at 200°C overnight. Gel electrophoresis equipment used for the separation of RNA was pre-soaked in 0.2 M NaOH for 30 minutes and washed extensively in distilled water to remove potential RNase contamination.

All manipulations were carried out on ice unless otherwise stated. The initial extraction procedures for the isolation of nucleic acid were carried out in a cold room at 4°C.

The concentration and quality of nucleic acid was determined using a Beckman DU® Series 70 spectrophotometer that scanned between 220-300 nm. At 260 nm the concentration of RNA and DNA at 1 OD unit is equivalent to 40 µg/ml and 50 µg/ml respectively. Samples of uncontaminated RNA and DNA have OD ratios at 260/280 near 1.8 and 2.0 respectively (Sambrook *et al.* 1989).

2.4.2 Isolation of nucleic acids

2.4.2.1 Isolation of plasmid DNA from *Escherichia coli*

A sterile tooth pick was inserted into the centre of a plasmid bearing bacterial colony and used to inoculate 5 ml of LB medium containing antibiotic where appropriate. These cultures were then incubated in a New Brunswick shaking incubator at 200 rpm and 37°C for 12 to 16 hours. For small scale plasmid DNA preparations 1 ml of the culture was prepared according to the alkaline SDS lysis method of Birnboim and Doly (1979).

For large scale plasmid DNA preparations the 5ml culture was used to inoculate 500ml of LB medium containing 100 mg/l of ampicillin or 50 mg/l of kanamycin. These cultures were shaken for a further 12 to 16 hours as above. The plasmid DNA was then isolated according to the lysozyme/triton lysis method of Katz *et al.* (1973) and purified in CsCl/EtBr density gradients (Radloff *et al.* 1967). The supercoiled DNA fraction was collected by side puncture and banded again. The DNA was again collected by side puncture, the ethidium bromide removed by repeated extraction with sodium chloride saturated 1-butanol and the DNA/CsCl diluted with 2 volumes of Milli-Q® Plus water.

The plasmid DNA was precipitated from solution in 70% ethanol (Sambrook *et al.* 1989) to separate the DNA and CsCl. The resulting pellet was washed in 70% ethanol, resuspended in TE buffer (10 mM TrisHCl, 0.1 mM EDTA pH 8.0.) and precipitated in 70% ethanol to remove any

residual salt. After a final wash in 70% ethanol the DNA was resuspended in TE buffer to a final concentration of 1 mg/ml and stored at 4°C.

2.4.2.2 Isolation of RNA from plant tissue

The following method was used to isolated RNA from up to 3 g of plant tissue and was adapted from Logemann *et al.* (1987). Plant material (either fresh or previously stored at -80°C) was ground to a fine powder using a pestle and mortar with liquid nitrogen. Using a sterile spatula the frozen tissue was transferred to a 30 ml polypropylene tube to which was added 4 ml (per g of tissue) of extraction buffer (8 M guanidine hydrochloride, 20 mM MES, 20 mM EDTA, 50 mM β -mercaptoethanol pH 7.0). The tissue was then stirred thoroughly with a sterile glass rod and left for up to 30 minutes.

An equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1 v/v, the phenol having been saturated with 100 mM TrisHCl pH 8.0 and containing 0.1% (w/v) 8-hydroxyquinoline) was added to each tube and then vortexed for 20 sec. Centrifugation was then carried out at 10,000 x g for 10 minutes in a Beckman JS-13.1 rotor and the supernatant was transferred to a new tube. The phenol extraction was repeated and then followed by an extraction with chloroform : isoamyl alcohol (24 : 1 v/v). To dilute the guanadine salt 1 volume of sterile Milli-Q® Plus water was added to each aqueous phase.

Ethanol precipitation of the nucleic acids from the aqueous phase was carried out with the addition of 0.1 volume of 3 M NaOAc pH 5.2 and 2 volumes of ethanol (stored at -20°C) and storage at -80°C for 30 minutes (or -20°C overnight). Sedimentation of the precipitate was carried out by centrifugation at 10,000 x g for 10 minutes using a Beckman JS-13.1 rotor. The nucleic acid pellet was washed with 70% ethanol, resuspended in sterile Milli-Q® Plus water, ethanol precipitated as above, washed in 70% ethanol, air dried and resuspended in sterile Milli-Q® Plus water to a final concentration of approximately 1 mg /ml and stored at -80°C.

The isolated nucleic acid was used routinely for northern blot analysis of RNA, but for primer extension and RNase protection the DNA was removed. This was achieved by preferentially precipitating the RNA from the nucleic acid in 2 M LiCl stored overnight at 4°C followed by

centrifugation at 10,000 × g for 10 minutes. The RNA pellet was washed in 70% ethanol, resuspended in sterile Milli-Q® Plus water and ethanol precipitated a further two times to remove residual salt before being resuspended in sterile Milli-Q® Plus water and stored as above.

For RNA sequencing the RNA was further enriched for polyA⁺ mRNA using Hybond-MAP™ (messenger affinity paper) following the manufacturers recommended protocol (Amersham International Plc.).

2.4.2.3 Isolation of DNA from plant tissue

Approximately 1 g of plant tissue was ground to a fine powder using a pestle and mortar with liquid nitrogen. The tissue was then transferred to a 15 ml polypropylene tube containing 6 ml of extraction buffer (42% (w/v) Urea, 0.3 M NaCl, 50 mM TrisHCl pH 8.0, 20 mM EDTA, 0.4% (w/v) sodium dodecyl sulphate (SDS)). An equal volume of phenol : chloroform : isoamyl alcohol (24 : 24 : 1 v/v) was added, mixed by gentle shaking and followed by centrifugation at 10,000 × g for 10 minutes. The aqueous phase was then removed and the nucleic acid was precipitated with the addition of 0.5 volumes of 7.5 M NH₄Ac and 0.6 volumes of isopropanol followed by centrifugation at 10,000 × g for 5 minutes. The air dried pellet was then resuspended in 400 µl of TE buffer.

The solution of nucleic acid was then digested with 5µl of DNase-free RNase A (10 mg /ml stock solution) at 37°C for 30 minutes. Phenol/Chloroform extractions were then repeated until the supernatant appeared to be clear of contaminants. After an ethanol precipitation the DNA pellet was washed with 70% ethanol, air dried and resuspended in 200µl of TE buffer.

2.4.3 Isolation of proteins from the maize nucleus

2-day-old dark-grown maize seedlings were harvested into a beaker on ice. The tissue was ground in a pestle and mortar using 20 ml (per g of tissue) of the grinding buffer (77% Honda buffer (3% Ficoll T400, 7% dextran T40, 30mM TrisHCl pH 8.5, 25 mM EDTA, 0.5% Triton X-100), 460 mM sucrose, 10 mM β-mercaptoethanol, 2 mM spermine, 0.5 mM spermidine). After stirring well the mixture was filtered through 4 layers of muslin soaked in grinding buffer and then through 1 piece of mira cloth. The filtrate was

centrifuged for 5 min at 4000 x g in the JA-20 rotor at 0°C. The supernatant was discarded and the pellets were resuspended in 10 ml of resuspension buffer (50 mM TrisHCl pH 8.5, 5 mM MgCl₂, 25% glycerol, 10 mM β-mercaptoethanol). It was then centrifuged for 10 minutes at 6000 x g in the JA-20 rotor at 0°C. The supernatant was discarded and the pellets were resuspended in 0.5 ml of salt buffer (50 mM TrisHCl pH 7.8, 20% glycerol, 0.42M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM β-mercaptoethanol, 1.5 mM MgCl₂). The resuspended pellets were then sonicated three times at an amplitude of 6 microns for 2 seconds with 10 seconds between each pulse. The mixture was then stirred in the cold room for 1 hour with a microflea and then centrifuged for 25 minutes at 37,000 x g in the TL100.3 rotor at 0°C. The supernatant was then dialysed for at least 10 hours with a buffer of 50 mM TrisHCl pH 7.8, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 1 mM β-mercaptoethanol. The protein extract was then concentrated with an Amicon Centricon-10 centrifuging at 4000 x g in a JA-20 rotor at -5°C until the desired volume was obtained (minimum of 50 μl).

2.4.4 Radiolabelling of nucleic acids for hybridisation studies

2.4.4.1 Random primer extensions

Isolated fragments (section 2.4.5) of DNA were labelled, with α[³²P]dCTP, by oligonucleotide random priming, as described by Feinberg and Vogelstein (1984), using the Amersham Multiprime DNA labelling system. Unincorporated nucleotides were separated from labelled DNA fragments on a Pharmacia NICK-column which contained Sephadex' G50.

2.4.4.2 RNA probes

Plasmid DNA was linearised with an appropriate restriction enzyme using standard recombinant DNA techniques (Sambrook *et al.* 1989). RNA probes were synthesised with α[³²P]UTP using the Promega Riboprobe® gemini II system as described in the Promega *Protocols and applications guide*.

A modified method was used to synthesise RNA probes for RNase protection experiments to ensure the probes were full length (Krieg 1990). The concentration of unlabelled UTP in the reaction cocktail was increased

from 12 nM to 50 nM, the other NTP's being 500 nM. Also the incubation with the RNA polymerase was carried out at 4°C for 1 hour rather than at 37°C for 30 minutes.

2.4.4.3 5' end labelling of oligonucleotides

Oligonucleotides, synthesised by Oswell DNA Services, were end labelled with T4 polynucleotide kinase using standard procedures (Sambrook *et al.* 1989). Each reaction contained 10 pmol of oligonucleotide and γ [³²P]dATP (5000 Ci/mmol) and was incubated at 37°C for 30 minutes. The end labelled oligonucleotides were separated from the unincorporated nucleotides by electrophoresis through a denaturing 19% (w/v) polyacrylamide gel as described by Sambrook *et al.* (1989).

2.4.4.4 End labelling DNA fragments for DNA-protein interaction assays

End labelling of DNA was carried out using klenow and infilling restriction sites with 5' overhangs. Each 25 μ l reaction mixture consisted of 10 mCuries of α [³²P]dCTP and other constituents at the following concentration, 80mM each of dATP, dTTP, dGTP, 50mM TrisCl pH 7.2, 10 mM MgSO₄, 0.1 mM DTT, 100 g/ml BSA and 10 units of klenow. This was left at room temperature for 30 minutes and the reaction was stopped with 1 μ l of 0.5 M EDTA.

2.4.5 Nucleic acid hybridisation

2.4.5.1 Southern blot analysis

Digested plasmid DNA was fractionated by electrophoresis through a horizontal 1.0 % (w/v) agarose gel buffered with 0.5xTBE. The gel was electrophoresed for less than 4 hours using 0.5xTBE as an electrophoresis buffer. The fractionated DNA was transferred to Hybond-N using a low pressure vacuum generated by an LKB 2016 VacuGene Vacuum Blotting System. The protocol described in the equipment manual involves treating the agarose gel sequentially with depurination solution, denaturation solution and neutralising solution. This is followed by transfer for 1 hour with 20xSSC. The air dried nylon membranes were UV crosslinked using a Stratalinker' UV crosslinker 1800 (Stratagene) set at 120 mJoules/cm².

Prehybridisation was carried out in a rotary oven (Hybaid) set at 42°C in a buffer of 50% (v/v) formamide, 5xSSC, 0.5% SDS, 5xDenhardtts solution (100x is equivalent to 2% (w/v) ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) bovine serum albumin (BSA)) and 0.5 mg/ml single stranded salmon sperm DNA for at least 1 hour. Hybridisation was routinely carried out overnight in the conditions above with the addition of the relevant DNA probe.

Washes were also carried out at 42°C the most stringent being 1xSSC for 30 minutes. Filters were then dried and exposed to Cronex-4® X-ray film, which had been preflashed as described by Laskey and Mills (1975), at -70°C with intensifying screens.

2.4.5.2 Northern blot analysis

Total nucleic acid, equivalent to 8 µg of RNA, was fractionated by electrophoresis through a horizontal 1.3% (w/v) agarose, 3.7% (v/v) formaldehyde denaturing gel buffered with 1xMOPS at pH 8.0. Each RNA sample included 1xMOPS pH 8.0, 40 µg/ml EtBr, 1% (v/v) formaldehyde, 30% (v/v) formamide (Barkan and Martienssen, 1991) and was incubated at 70°C for 2.5 minutes before being loaded onto the gel. The gel was electrophoresed for less than 4 hours using 1xMOPS pH 7.0 as an electrophoresis buffer.

The fractionated RNA was transferred to Hybond-N using 20xSSPE as described in the manufacturers protocol (Amersham International Plc.). The air dried nylon membranes were UV crosslinked using a Stratalinker' UV crosslinker 1800 (Stratagene) set at 120 mJoules/cm².

Prehybridisation was carried out in a rotary oven (Hybaid) set at 55°C in a buffer of 50% (v/v) formamide, 0.75 M NaCl, 0.15 M TrisHCl pH 8.0, 0.01 M EDTA, 0.1% SDS, 1xDenhardtts solution (100x is equivalent to 2% (w/v) ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) bovine serum albumin (BSA)) and 0.5 mg/ml single stranded salmon sperm DNA for at least 1 hour. Hybridisation was routinely carried out overnight in the conditions above with the addition of the relevant RNA probe.

Washes were also carried out at 55°C the most stringent being 50 mM NaCl, 5 mM TrisHCl pH 8.0, 0.5 mM EDTA, 0.1% SDS, 0.1% tetra-sodium

pyrophosphate. Washed filters were then treated with RNase A buffer (20 µg/ml RNase A, 0.5 M NaCl, 10 mM TrisHCl pH 8.0, 1 mM EDTA) at 37°C for 30 minutes which efficiently removes any non specific binding. The filters were then washed twice in the same buffer without RNase A at 37°C for 30 minutes. Filters were then dried and exposed to Cronex-4® X-ray film, which had been preflashed as described by Laskey and Mills (1975), at -70°C with intensifying screens.

2.4.5.3 Screening λ gt10 library

End labelled oligonucleotides (section 2.4.4.3) were used as probes whose T_m values were calculated using the equation $T_m = 2(A+T) + 4(G+C)$ (Itakura *et al.*, 1984). The prehybridisation, hybridisation and washes were then carried out at a temperature 5°C below the calculated T_m value. The prehybridisation and hybridisation buffers were identical (6xSSPE, 1xDenhardtts solution, 0.1% SDS, 0.5 mg/ml single stranded salmon sperm DNA) except the hybridisation buffer included the end labelled oligonucleotide prepared as in section 2.4.4.3. All washes were carried out in 6xSSPE.

2.4.6 Creation of subclones

Fragments of DNA were isolated for subcloning by fractionating plasmid DNA, which had been digested with appropriate restriction enzymes, in a 1xTBE agarose electrophoresis gel. The fragment for subcloning was visualised, after staining with EtBr, on a UV transilluminator (Ultra-violet Products Ltd.) and excised from the gel. The DNA fragment was then recovered from the agarose using a Biotrap (Schleicher and Schuell GmbH). Subclones, in the various plasmids described in this thesis, were then created using standard techniques (Sambrook *et al.*, 1989).

2.4.7 Nucleotide sequence analysis

2.4.7.1 DNA sequencing

Plasmid DNA was used as the sequencing template for all sequencing reactions and was prepared using a method modified from the alkaline-lysis mini-preparation method (Stephen *et al.*, 1990). Sequencing primers used were either the reverse primer or -40 primer (both specific for the pBS M13+ and pBluescript vectors used to clone DNA for sequencing) or

synthetic oligonucleotides complementary to specific regions of the cloned DNA sequence. Two sequencing kits were used; the T⁷Sequencing[®] Kit and the Sequenase[®] Version 2.0 (section 2.2.2) both of which incorporate α [³⁵S]dATP and are based on the dideoxy chain termination method of Sanger *et al.* (1977).

Labelled products were fractionated by electrophoresis in a 5% (w/v) polyacrylamide gel as described in Sambrook *et al.* (1989). All gels were dried, then exposed to Cronex-4[®] X-ray film at room temperature.

2.4.7.2 RNA sequencing

60 μ g of RNA (section 2.4.2.2) was co-precipitated with 10⁵ cpm of end labelled oligonucleotide primer (section 2.4.4.3) with 0.1 volumes of 3 M NaOAc and 2.5 volumes of ethanol (stored at -20°C) and storage at -80°C for 30 minutes. After 2 washes with 70% ethanol the pellet was air dried and resuspended in 20 μ l of sterile Milli-Q[®] Plus water.

To anneal the oligonucleotides to the RNA template the solution was incubated at 90°C for 2 minutes, 5 μ l of annealing buffer (2 M NaCl, 50 mM PIPES pH 6.4) was added followed by incubation at 50°C for 15 minutes.

A dideoxy reaction cocktail was made for each nucleotide such that the final reaction conditions were 50 mM TrisHCl pH 8.3, 10 mM DTT, 6 mM MgCl₂, 300 mM of each dNTP, 160 mM of 1 ddNTP (G,A,T or C), 25 units reverse transcriptase. 4 μ l of the annealing mix was then added to 16 μ l of each of the four dideoxy cocktails. The reaction proceeded for 3 hours at 42°C after which the reaction products were diluted with TE buffer and precipitated with 2.5 volumes of ethanol. The pellet was then resuspended with 50 μ l of 0.3 M NaOH, 1 mM EDTA and incubated at 65°C for 30 minutes. The reaction was then neutralised with 60 μ l of 1 M TrisHCl pH 7.5, precipitated with 2.5 volumes of ethanol, washed with 70% ethanol and air dried.

The pellet was resuspended in formamide loading buffer (80% (v/v) formamide, 10 mM EDTA, 1 mg /ml xylene cyanol FF, 1 mg /ml bromophenol blue) and fractionated by electrophoresis in a 5% (w/v) denaturing polyacrylamide gel, along with sequencing reaction size markers which used the same oligonucleotide primer, as described in

Sambrook *et al.* (1989). All gels were dried, then exposed to Cronex-4® X-ray film at -70°C with intensifying screens.

2.4.8 Primer extension analysis

50 µg of RNA (section 2.4.2.2) was co-precipitated with 10⁵ cpm of end labelled oligonucleotide primer with 0.1 volumes of 3 M NaOAc and 2.5 volumes of ethanol (stored at -20°C) and storage at -80°C for 30 minutes. After 2 washes with 70% ethanol the pellet was air dried and resuspended in 8 µl of sterile Milli-Q® Plus water.

To anneal the oligonucleotides to the RNA template the solution was incubated at 90°C for 2 minutes, 2 µl of annealing buffer (2 M NaCl, 50 mM PIPES pH 6.4) was added followed by incubation at 50°C for 15 minutes. The primer extension reaction was then carried out with the addition of the reaction constituents as a cocktail giving a final concentration of 50 mM TrisHCl pH 8.3, 10 mM DTT, 6 mM MgCl₂, 1 mM of each dNTP, 25 units reverse transcriptase to a final volume of 100 µl. The reaction proceeded for 3 hours at 42°C after which the reaction products were precipitated with 2.5 volumes of ethanol. The pellet was then processed as above in section 2.4.7.2.

2.4.9 RNase protection analysis

An RNA probe was made (section 2.4.4.2) of the sequence of interest and treated with RNase-free DNase to remove all plasmid DNA. 150 µg of RNA (section 2.4.2.2) was ethanol precipitated with 0.1 volumes of 3 M NaOAc and 2.5 volumes of ethanol (stored at -20°C) and storage at -80°C for 30 minutes. After 2 washes with 70% ethanol the pellet was air dried and resuspended in 30 µl of hybridisation buffer (40mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% (v/v) formamide) containing a small molar excess of RNA probe. The hybridisation mixture was incubated at 85°C for 10 minutes and transferred to another incubator at 55°C for 8-12 hours.

The hybridisation mixture was cooled to room temperature and treated with 300 µl of an RNase digestion mixture (300 mM NaCl, 10 mM TrisHCl pH 7.4, 5 mM EDTA, 0.4 µg/ml RNase T1, 8 µg/ml RNase A) at 30°C for 30 minutes. The addition of 20 µl of 10% SDS and 10 µl of a 10 mg/ml solution of proteinase K was followed by a further incubation at 37°C for

30 minutes. After a thorough Phenol : Chloroform extraction the protected probe was precipitated with 2.5 volumes of ethanol.

The pellet was resuspended in formamide loading buffer (80% formamide, 10 mM EDTA, 1 mg /ml xylene cyanol FF, 1 mg /ml bromophenol blue) and fractionated by electrophoresis in a 5% (w/v) denaturing polyacrylamide gel, along with sequencing reaction size markers , as described in Sambrook *et al.* (1989). All gels were dried, then exposed to Cronex-4[®] X-ray film at -70°C with intensifying screens.

2.4.10 DNA-protein interaction assay

These assay conditions are based on a protocol described by Hennighausen and Luban (1985). Up to 10 µg of nuclear extract (section 2.4.3) was mixed with 1 µg of poly dIdC and approximately 4 ng of end labelled DNA (section 2.4.4.4) in binding conditions of 10 mM HEPES pH 7.0, 40 mM NaCl, 10 mM MgCl₂, 1 mM DTT and 0.2 mM EDTA in a final volume of 100 µl. This sample was then incubated for 20 minutes at room temperature, filtered through nitrocellulose, at a rate of 2 ml/minute, using a dot blot apparatus and washed with 2 ml of binding buffer. The DNA bound to the nitrocellulose was eluted with a solution of 0.1% SDS, 10mM EDTA, 0.3 M NaOAc pH 5.2, 0.35 mg/ml proteinase K by shaking for 1 hour at 37°C. After phenol:chloroform extraction and ethanol precipitation the DNA was resuspended in 0.1% SDS, 11.25 mM EDTA, 50 mM TrisHCl, 50 mM boric acid, 3% (w/v) ficoll and 0.02% (w/v) bromophenol blue. The DNA was electrophoresed through either a 2% (w/v) agarose gel or a 4% (w/v) polyacrylamide gel using 1xTBE as the electrophoresis buffer.

2.5 COMPUTER ANALYSIS

Assembly and analysis of nucleotide and derived amino acid sequence data was carried out using the programs (Devereux *et al.*, 1984) of the University of Wisconsin Genetics Computer Group through the VAX 8000 system at Edinburgh University.

CHAPTER 3

CHARACTERISATION OF THE PROMOTER UPSTREAM OF *ANT1* AND *ANT2*

3.1 INTRODUCTION

Prior to the work described in this thesis, the maize *ANT* genes had been studied in this laboratory for some time. This earlier work is summarised in section 3.2. The remainder of this chapter discusses experiments to identify regulatory elements upstream of the maize *ANT* clones. The following chapter discusses experiments concerned with the cloning of a cDNA clone for *ANT2* and an analysis of *ANT* gene structure. I have shown that some conclusions from the previous work on gene structure were incorrect due to a misinterpretation of the 5' analysis data. In fact, the 5' end of *ANT1* and *ANT2* are more complex than previously concluded. I have shown that at least two introns are present in the untranslated leader sequence of both and that there are multiple transcription initiation sites. The final results chapter discusses tissue specific and differential expression of the *ANT* genes. The coordinated expression of genes associated with oxidative phosphorylation was also analysed using RNA probes for *ANT* and ATP synthase (nuclear and mitochondrially encoded subunits).

3.2 PREVIOUS WORK

3.2.1 The isolation, sequence and structure of maize genomic and cDNA clones encoding the *ANT* protein

Previous work carried out in this laboratory, prior to this study, has shown that the *ANT* protein from maize is encoded in the nucleus by two genes (Baker and Leaver, 1985, Bathgate *et al.*, 1989). Both genes had been cloned from a maize genomic library and designated *ANT1* and *ANT2*. The two clones, which have sizes of 8.0 kb and 6.4 kb respectively, had been partially sequenced including all the open reading frame and a limited amount of the upstream (5') and downstream (3') sequences. For *ANT1* approximately 2 kb of the upstream region had not been sequenced while for *ANT2* the unsequenced upstream region was approximately 3 kb (Figure 3.1).



Figure 3.1

A summary of the *ANT* genomic and cDNA clones which had been isolated and the characterisation which had been completed prior to the start of this thesis.

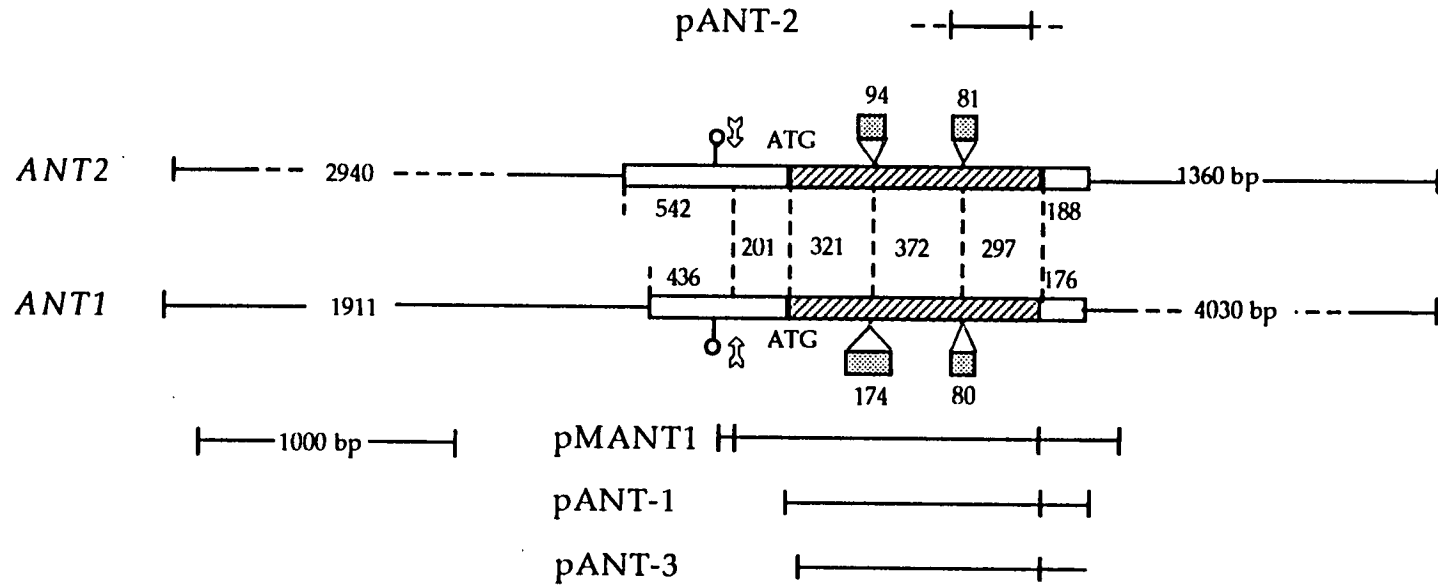
Genomic clones

The boxed regions in the genomic clones represent the DNA which has been sequenced whereas the regions denoted by a line are unsequenced. The numbers represent the sizes in base pairs. The *ANT1* genomic clone has an estimated size of 8 kb. The *ANT2* genomic clone has an estimated size of 6.4 kb. Both clones encode ANT proteins with a putative size of 330 amino acids. The putative TATA box, transcript initiation sites and translational initiation site are indicated according to the key below:

- ATG translational start site
- ☉ TATA box
- ⤴ transcript initiation site
- ▭ sequenced regions of the genomic clone
- ▨ open reading frame
- ▩ intron sequence
- unsequenced regions of the genomic clone

cDNA clones

The three *ANT1* cDNA clones and *ANT2* cDNA clone are shown in relation to the genomic clones. The solid lines represent the sequenced part of the clones and in the case for pMANT1, pANT-1 and pANT-3 indicate the 5' and 3' termini. The 64 bp sequence indicated at the 5' terminus of the pMANT1 clone represents the sequence which was not similar with the genomic sequence in *ANT1*.



In addition to the two genomic clones several cDNA clones of *ANT* had been isolated from a maize cDNA library. Three were identical to the sequence of *ANT1*, one of these was believed to be full length and has been designated pMANT1 (Baker, 1985; Winning *et al.* 1991). Only one short cDNA clone had been identified that showed sequence identity to *ANT2*. By comparing the cDNA sequences with the genomic sequences two introns were identified in both *ANT1* and *ANT2*. The sites of insertion in both genes are identical and the similarity of the second introns are 63/82 nucleotides. The first introns also show high similarity (63/98 nucleotides) but an additional 76 bp are present in the *ANT1* sequence. An ATG common to both genes had also been identified followed by an open reading frame encoding a 329 amino acid protein. All the information above regarding the *ANT* cDNA and genomic clones, the gene structure and the known genomic sequence before the present work was started is summarised in Figure 3.1.

3.2.2 Identification of the transcription initiation site

Several putative transcription initiation sites had been identified with primer extension experiments, using an oligonucleotide to a common sequence in *ANT1* and *ANT2*. The initiation sites had been confirmed with S1 nuclease and mung bean nuclease protection experiments (Figure 3.2). It was not known which of these initiation sites corresponded to which gene since transcripts from both *ANT1* and *ANT2* were present in the RNA samples studied (Bathgate *et al.*, 1989). The cDNA clone pMANT1 showed 100% similarity with the *ANT1* clone up to the putative initiation site (Figure 3.2). The first 64 bp of pMANT1 showed no sequence similarity with any of the 442 bp of the *ANT1* upstream region sequenced at the time and had been considered to be the result of a cloning artifact.

3.2.3 Expression of the *ANT* genes

The maize cDNA library used to isolate the *ANT* cDNA clones was made from polyA⁺ RNA isolated from 3-day-old dark-grown seedlings and was shown to have clones representing *ANT1* and *ANT2*. Thus it was assumed that both *ANT* genes were expressed at that stage of development. No differential probes had been available to substantiate this result by northern blot transcript analysis.

Figure 3.2

The upper sequence is the 5'-end of the cDNA clone pMANT1. The lower two of the three sequences are the regions from the genomic sequences of ANT1 and ANT2 complementary to pMANT1. The nucleotide numbering represents the distance from the 5'-end of the known sequence in the respective genomic clones as shown in figure 3.1. The predicted amino acid sequence from the ANT1 open reading frame is immediately below the nucleotide sequences. The predicted amino acid sequence for ANT2 is identical unless indicated by a residue below the the ANT1 amino acid sequence.

The putative TATA boxes have been underlined. The 5' termini of the ANT transcripts were mapped using three different techniques. The three primer extension products using the universal oligonucleotide (boxed sequences complementary to the nucleotides 238-256 bp in pMANT1) are indicated by arrows (\uparrow). The mung bean nuclease protection site is indicated by an open triangle (Δ) and the S1 nuclease protection site is indicated by a solid arrow (\blacktriangleright).

Oligonucleotides used for differential primer extension are boxed. The ANT1 specific oligonucleotide is complementary to the ANT1 sequence from 674-690 bp. The ANT2 specific oligonucleotide is complementary to the ANT2 sequence from 781-798 bp. No extension products using these oligonucleotides were defined.

Using a DNA probe, which is common to both *ANT1* and *ANT2* transcripts, derived from pANT-1 (Figure 3.1) a northern blot of RNA isolated from serial sections of 10 day-old maize leaves was probed. It was shown that *ANT* transcript steady state levels decrease up the leaf from the basal meristematic region and have an inverse relationship with transcript levels of photosynthetic genes (Bathgate *et al.*, 1989). This result was also confirmed using a similar wheat leaf developmental system (Topping, 1987).

3.3 RATIONALE

To understand the molecular basis of mitochondrial biogenesis in plants and investigate how this is influenced by environmental, metabolic and developmental signals, several nuclear genes which encode mitochondrial proteins have been cloned from maize. Two of these genes are *ANT1* and *ANT2* which encode adenine nucleotide translocators, the most abundant mitochondrial protein (Klingenberg, 1976, 1988).

The ANT protein has a high specificity for ADP/ATP nucleotides and mediates the exchange of these nucleotides between the cytosol and mitochondrial matrix. The supply of ADP to the matrix, mediated by ANT, may exert a significant control over the rate of oxidative phosphorylation at certain developmental stages (Groen *et al.*, 1982 and section 1.4.1.1). Consequently the regulation of these genes may be coordinated in response to various developmental changes, availability of metabolites or stress to satisfy the plant cell's energy requirements.

Approximately 2.5 kb and 3.5 kb of the DNA sequence upstream of the transcription initiation sites for *ANT1* and *ANT2* have been cloned. These regions were expected to contain the specific regulatory elements necessary for the expression of *ANT*. Using appropriate DNA-protein binding protocols I analysed these upstream regions for potential cis-acting elements and related trans-acting factors in an attempt to elucidate how these genes are regulated.

3.4 SUBCLONING THE *ANT* 5' REGIONS AND RESTRICTION ENDONUCLEASE MAPPING

A detailed restriction map of the genomic 5' regions of *ANT1* and *ANT2* was prepared to allow analysis of small, accurately defined DNA fragments from the promoter. To reduce the size of the DNA to be mapped the genomic 5' regions were subcloned from the original genomic clones. The first 3.8 kb (EcoRI-ClaI) from the 8.0 kb *ANT1* genomic clone was subcloned into pK18 (linearised with EcoRI-AccI) and named pKG15. The first 2.5 kb of this subclone is upstream of the transcription initiation site. The first 3.9 kb (EcoRI-PstI) from the 6.4 kb *ANT2* genomic clone was subcloned into pUC19 (linearised with EcoRI-PstI) and named pUG25. The first 3.6 kb of this subclone is upstream of the transcription initiation site (Figure 3.3).

Restriction mapping of pKG15 and pUG25 was achieved using the known positions of EcoRI and PstI, at the extreme 5' and 3' ends of the insert, as reference points. Additional mapping using partial digests of end labelled fragments allowed the order of the smaller fragments to be determined (Figure 3.3). This map has been confirmed, with a few modifications, from the sequence data obtained in section 4.7.

3.5 TRANSLATIONAL (IN FRAME) FUSIONS BETWEEN THE TWO *ANT* PROMOTERS AND THE GUS REPORTER GENE

In order to analyse *ANT1* and *ANT2* specific expression in transgenic plants fusions between the two *ANT* promoters and the GUS reporter gene were constructed. The promoter regions from both the *ANT* genes were fused to the GUS reporter gene. The initiation ATG from *ANT* and the sequence encoding the first 39 amino acids were fused to the GUS open reading frame to form a contiguous sequence (a translational fusion).

The subclone pKG15 (above) was digested with the restriction enzyme PvuII and the 2.7 kb fragment, which included the promoter region, was isolated using the procedures described in section 2.4.6. This fragment has 88 bp from the pK18 vector sequence at the 5' end and 117 bp of *ANT* open reading frame at the 3' end. The GUS gene was derived from the plasmid pBI201 which includes a polylinker upstream of the GUS open reading frame and a termination signal from the nopaline synthase (NOS)

Figure 3.3

A detailed restriction map of the 5' regions from the 5'-end of each genomic clone to the start of the ANT open reading frames.

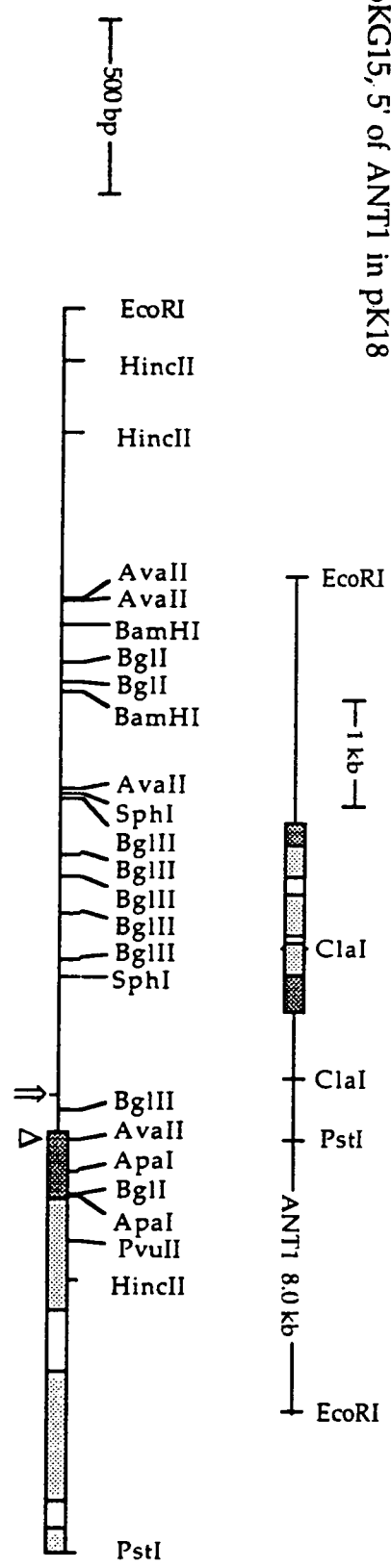
A The 5' region of *ANT1* from EcoRI to ClaI (approximately 3.8 kb) was subcloned into the pK18 cloning vector which had been linearised with EcoRI and AccI. This plasmid was termed pKG15. The ClaI site was destroyed with the ligation of the insert and plasmid and the PstI site at the 3' end of the *ANT1* specific insert is the PstI in the MCS of pK18.

B The 5' region of *ANT2* from EcoRI to PstI (approximately 3.9 kb) was subcloned into the pUC19 cloning vector which had been linearised with EcoRI and PstI. This plasmid was termed pUG25.

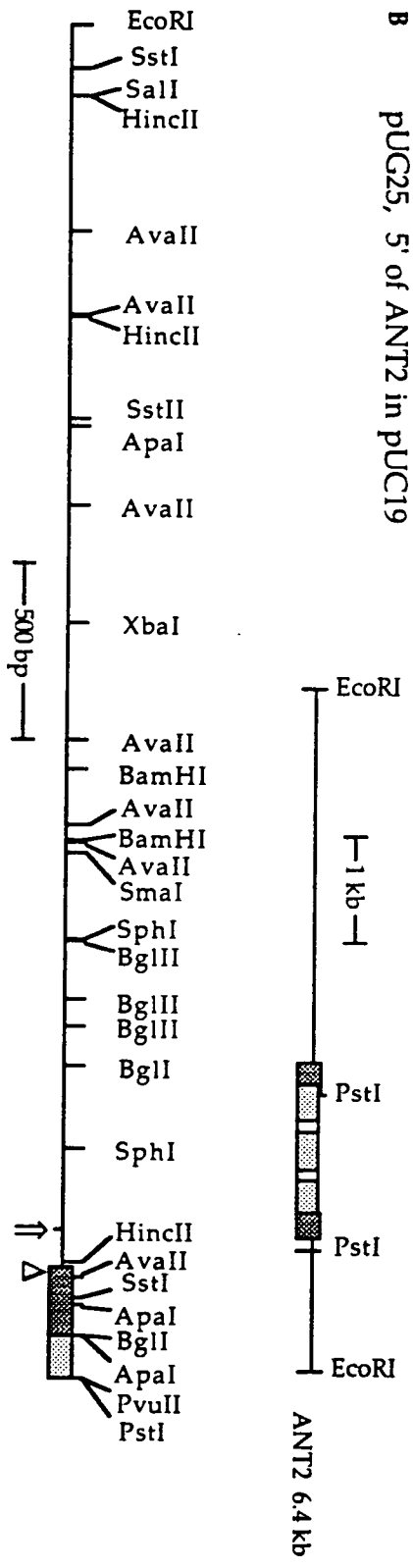
The symbols in the diagram are defined in the key below:

- ↑ TATA box
- Δ transcript initiation site
- intron sequence
- ▨ exon sequence
- 5' and 3' untranslated region:

A pKG15, 5' of ANT1 in pK18



B pUG25, 5' of ANT2 in pUC19



gene at the 3' end of the GUS sequence in a pUC19 based vector. Three versions of the plasmid (pBI201.1, 201.2, 201.3) provide the three reading frames of GUS relative to the polylinker sites. Version pBI201.2 allows an inframe fusion of *ANT* (PvuII) with *GUS* (SmaI in the polylinker). Therefore the 2.7 kb PvuII fragment (*ANT* promoter) was subcloned into the pBI201.2 vector which had been linearised with SmaI. This *ANT1/GUS* fusion was designated pG1-GUS (Figure 3.4). The PvuII/SmaI fusion junction was confirmed by sequencing with an oligonucleotide primer complementary to a sequence in the *GUS* open reading frame (data not shown).

The above cloning strategy was also used to construct an *ANT2/GUS* fusion clone by ligating the 3.8 kb PvuII fragment from pUG25 into pBI201.2 linearised with SmaI. This fusion clone was designated pG2-GUS (Figure 3.4).

3.6 ENHANCERS AND THE UPSTREAM REGION OF *ANT*

Enhancers activate promoter activity and are specific upstream sequences that are independent of position and orientation (Atchison, 1988). They regulate gene expression in association with trans-acting proteins and are often located within 1 kb of the transcription initiation site although some enhancer elements have been found further upstream (Stougaard *et al.*, 1987, Deikman and Fisher, 1988). The DNA-protein interactions between the *ANT1* promoter region and a maize nuclear protein extract were studied in an attempt to identify enhancer elements upstream of *ANT1*. To preclude the chance of missing enhancer sequence further than 1 kb upstream the whole of the available promoter sequence was assayed for any DNA-protein interaction.





Initially it was decided to only use upstream sequences from the *ANT1* promoter region since it can be digested into small fragments suitable for DNA-protein interaction using the restriction enzyme Sau3AI. Although there is more upstream sequence (1.1 kb) in the *ANT2* clone the restriction sites are not as convenient to release evenly spaced small fragments. Additionally it was considered unlikely that any enhancer is further upstream than the 2.5 kb limit of the *ANT1* clone.

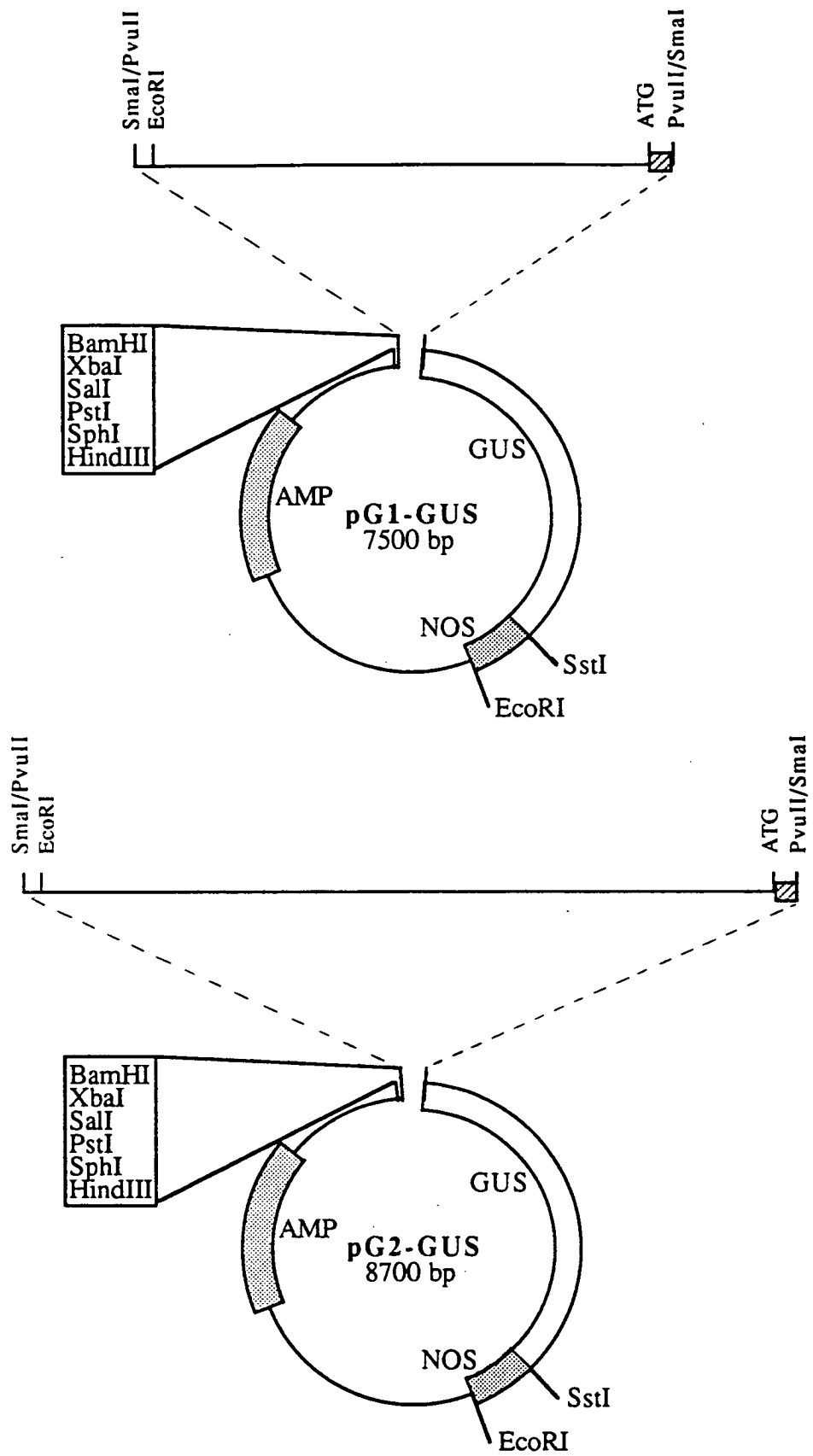
Figure 3.4

This map indicates the strategy for constructing the translational (in frame) fusions between the two putative ANT promoters and the GUS reporter gene. For both ANT genes the first 39 amino acids were fused to the GUS open reading frame to form a contiguous sequence.

PvuII fragments were isolated from the pKG15 and pUG25 plasmids. The PvuII fragments contain approximately 90 bp of the vector sequence at the 5' end (between the PvuII and EcoRI), putative promoter sequences in the middle and the first 39 amino acid codons from the ANT open reading frame at the 3' end. The EcoRI to PvuII 3'-ends of the PvuII fragments are *ANT1* (2.7 kb) and *ANT2* (3.9 kb) specific genomic sequences. These PvuII fragments were ligated into pBI201.2, which had been linearised with SmaI, to form the translational fusion between the ANT and GUS coding sequences. The *ANT1/GUS* translational fusion was termed pG1-GUS. The *ANT2/GUS* translational fusion was termed pG2-GUS.

The boxed endonuclease restriction enzymes indicate the restriction sites in the MCS which is 5' of the ANT promoter insertion. The symbols used in this figure are defined in the key below:

	ANT open reading frame
	GUS open reading frame
	NOS termination sequence
	Ampicillin resistance gene



Enhancers by definition are positive acting and the trans-acting proteins which interact with the enhancers are likely to be at their most abundant in tissues with active transcription (Atchison 1988). A nuclear protein extract was isolated from 2-day-old dark-grown maize seedlings and was used for the DNA-protein interaction assays. Protein from the dark-grown seedlings was used since it is known that both *ANT1* and *ANT2* transcripts are present at high steady state levels in this tissue (section 3.2.3). It is likely that high steady state levels coincide with active transcription. Therefore, when studying protein interactions with putative enhancers upstream of *ANT1* this is the logical tissue to use for a protein extract.

Although enhancers are common upstream elements that regulate the transcription of genes in a positive manner there are also regulatory elements termed silencers ("negative enhancers") that regulate the transcription in a negative manner (Brand *et al.*, 1985). Such elements serve as binding sites for trans-acting proteins that will be abundant in tissues where there is either no or low levels of transcription. For the *ANT* gene such factors would not be detected in the protein extract from dark-grown seedlings. It is possible that only silencers regulate *ANT* gene expression although genes are generally regulated using a combination of different enhancers and silencers. The experiments described in this chapter attempted to identify enhancers that function in dark-grown seedlings upstream of *ANT1* but were unlikely to identify silencers or other enhancers that function in different tissues.

3.7 DNA-PROTEIN INTERACTIONS BETWEEN THE *ANT1* PROMOTER REGION AND A MAIZE NUCLEAR PROTEIN EXTRACT

3.7.1 Isolation of the maize nuclear protein extract

The maize nuclei were isolated and washed with 0.42 M NaCl to extract the nuclear proteins as described in section 2.4.3. The histone proteins, which bind non-specifically to DNA, should not dissociate from the DNA at a salt concentration of 0.42 M but the trans-acting proteins should readily dissociate (Dignam *et al.*, 1983). The extract contains proteins of varying molecular weight; the most prominent had molecular weights of

between 10-20 kDa which may be histones (hence the extract may contain non-specific binding activities) (Figure 3.5).

3.7.2 DNA-protein interactions

The promoter region from *ANT1* was isolated from pKG15 by digesting the plasmid DNA with the restriction enzymes *EcoRI* and *PvuII*. The appropriate 2.8 kb fragment was separated by electrophoresis through an agarose gel and isolated using the Biotrap as described in section 2.4.6. The DNA fragment containing the promoter region was then digested with *Sau3A* into 16 small fragments ranging from 50 bp to 610 bp which were end labelled with α [³²P]dCTP as described in section 2.4.4.4.

The end labelled promoter fragments were incubated with the nuclear protein extract as described in section 2.4.10. The incubation mixture was filtered through a nitrocellulose membrane. The conditions allowed the DNA fragments to pass through the nitrocellulose but the proteins to bind to the nitrocellulose. Any DNA-protein complexes formed during the incubation should also bind to the nitrocellulose via the protein component of the complex. The end labelled DNA fragments bound to the nitrocellulose were eluted from the membrane, separated using gel electrophoresis and visualised using autoradiography (section 2.4.10).

If specific DNA-protein complexes are formed one or more of the 16 end labelled DNA fragments will bind to the nitrocellulose. If non-specific DNA-protein complexes are formed all of the 16 end labelled DNA fragments will bind to the nitrocellulose. The object of the assay is to distinguish between non-specific and specific binding, and to identify which DNA fragments are specifically bound. DNA fragments that form specific DNA-protein complexes may contain enhancer or promoter elements.

3.7.3 Eliminating non-specific binding

The nuclear protein extracts still contained a histone component which will bind to the DNA fragments non-specifically. To reduce or eliminate non-specific binding 1 μ g of synthetic poly dIdC was added to the incubation mix as a competitor for all non-specific DNA-protein interactions. The DNA binding proteins in the nuclear extract initially

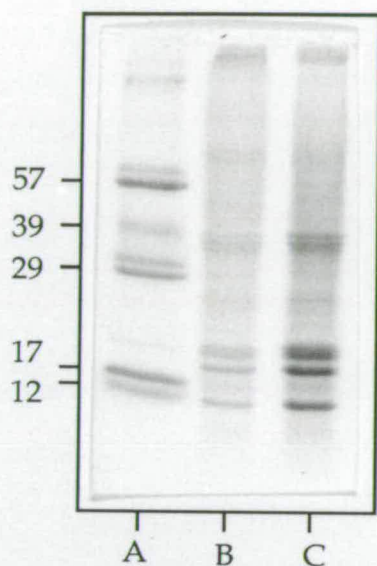


Figure 3.5

Proteins from a nuclear extract were isolated from 48 hr old dark-grown maize seedlings. The proteins were fractionated by electrophoresis in a 16% (w/v) SDS-polyacrylamide gel. The numbers to the left of the figure are the marker sizes and have the units $Mr \times 10^3$. Lane A contains the markers. Lanes B and C are nuclear protein extracts which were isolated by the same method (section 2.4.3) in two different experiments.

bind non-specifically to the poly dIdC and in theory specific trans-acting proteins in the nuclear extract then preferentially bind to the end labelled DNA fragments that contain specific binding sites for these proteins.

3.7.4 Candidate DNA fragments that contain promoter or enhancer elements

Using the assay as described in section 2.4.10 three DNA fragments were detected that appeared to bind specifically to proteins in the nuclear extract (Figure 3.6B). These fragments were localised -2440 to -1830, -1830 to -1590 and -390 to -50 bp upstream of the transcription initiation site and were identified as A, C and B respectively (Figure 3.6).

The extra bands between and above the A and B fragments in figure 3.6B are due to partial digestion of the 2.8 kb promoter region with Sau3A.

Enhancer elements at a location of -2440 to -1590 are not common but a recent publication by Li *et al.* (1990) reports the presence of an enhancer element -3.1 to -2.6 kb upstream of the human adenine nucleotide translocator gene (*ANT1*). Fragment B, which is from -390 to -50, contains the putative TATA box which would be expected to bind specifically to proteins in the nuclear extract (Figure 3.2). Enhancer elements are also commonly located within 400 bp upstream of the transcription initiation site.

The sequences of fragments A, B and C (section 4.7) were compared with the enhancer sequences postulated to coordinately regulate genes involved in human mitochondrial oxidative phosphorylation (Li *et al.*, 1990, Tomura *et al.*, 1990 and Suzuki *et al.*, 1991). No sequence similarity to postulated human enhancer elements were present in the three fragments.

3.7.5 Are the DNA-protein complexes specific or non-specific interactions ?

Although the data in figure 3.6B suggest that three DNA fragments upstream from the regions of *ANT1* form DNA-protein complexes there is also evidence to suggest these could be non-specific interactions.

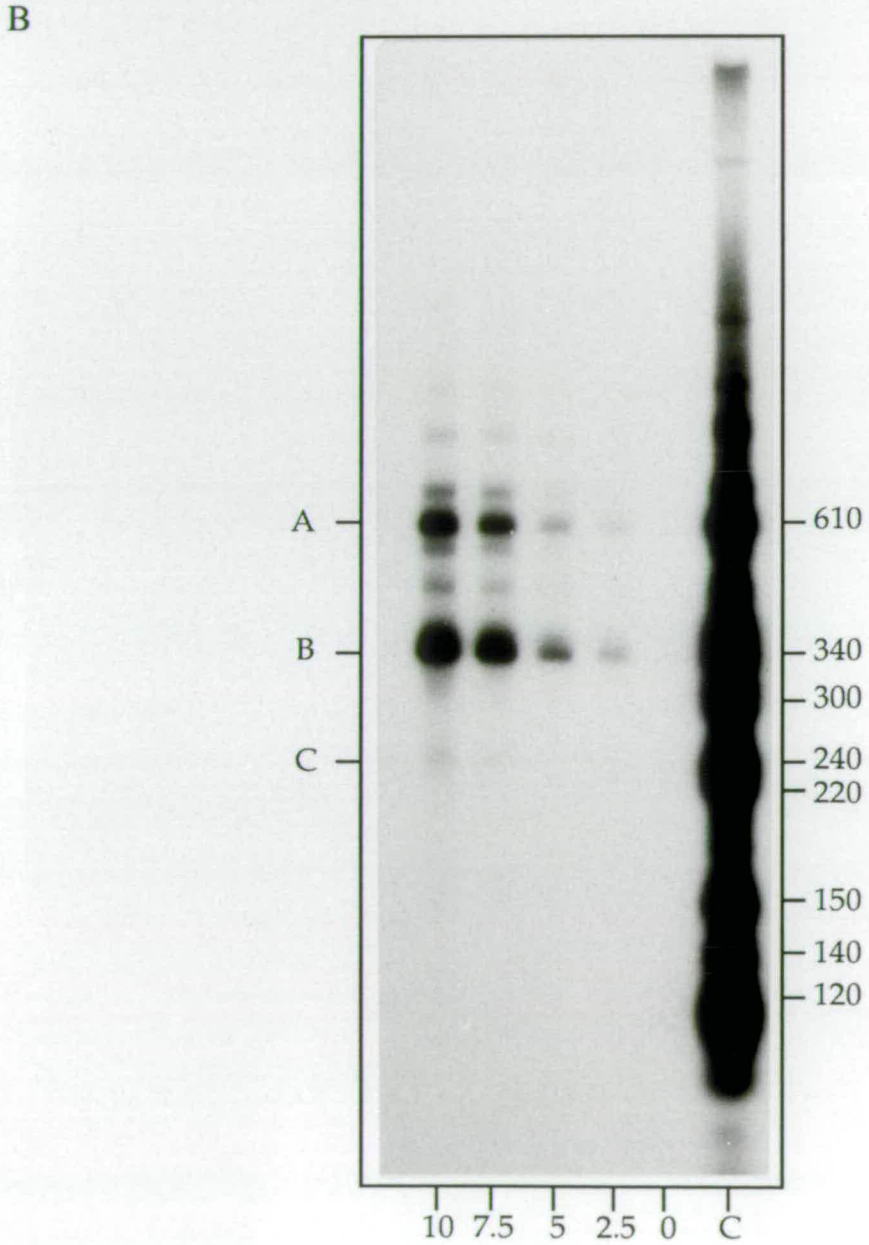
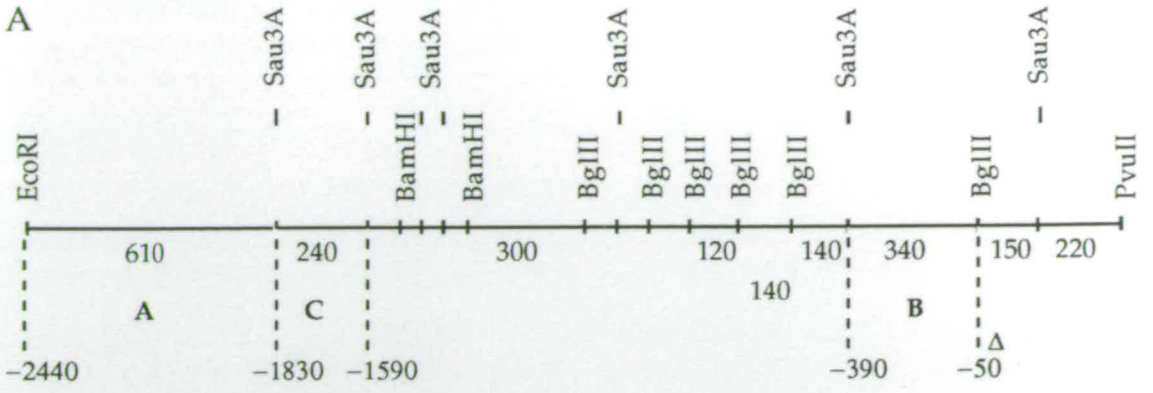
Figure 3.6

A The EcoRI-PvuII fragment containing the 5' end (2.8 kb) of the *ANT1* genomic clone was isolated from the pKG15 clone and digested with the endonuclease restriction enzyme Sau3A. The endonuclease restriction map in the figure indicates the position of the Sau3A restriction sites and the sizes of the resulting digestion products. BamHI and BglII also contain Sau3A endonuclease restriction sites. The Sau3A DNA fragments were endlabelled (section 2.4.4.4) and were used for DNA-protein interaction assays (as in section B below). Numbers below the dashed line indicate the position in the putative promoter region relative to the putative transcript initiation site (Δ). The letters A, B and C identify the location of the DNA digestion products shown in section B of this figure..

B This data is the result of a DNA-protein interaction assay (section 2.4.10). The basis of this assay is to incubate the nuclear protein extract with a suitable competitor DNA, in this case 1 μ g of poly dIdC, to remove non-specific binding. Endlabelled DNA fragments derived from the *ANT1* putative promoter region were added to the incubation. After 20 minutes the incubation mixture was filtered through nitrocellulose in conditions that caused protein but not nucleic acid to bind to the nitrocellulose. In addition to proteins, nucleic acids associated with protein bind indirectly to the nitrocellulose.

The 5 lanes from the left show the endlabelled DNA fragments which were eluted from the nitrocellulose and fractionated by electrophoresis in a 4% (w/v) SDS-polyacrylamide gel. The quantity of protein in each incubation decreases from the left as indicated by the numbers below the lanes. These numbers represent the quantity of nuclear protein extract (μ g) included in each incubation. Lane C is a 5th of the total endlabelled DNA which was included in each incubation.

The letters A, B and C to the left of the figure indicate the DNA fragments which were associated with proteins. The numbers to the right of the figure indicate the sizes of the endlabelled Sau3A DNA fragments in bp and correlate with the sizes indicated in section A of this figure.



Firstly, DNA-protein complexes have formed with the largest fragments. The larger DNA fragments are more likely to form a non-specific complex since a greater amount of DNA sequence is available for binding. To confirm this observation the 2.8 kb promoter fragment was digested with *MspI* and *TaqI*, as opposed to *Sau3A*, and end labelled. The two new sets of DNA fragments were incubated with a nuclear protein extract as before. A definite correlation between the size of the DNA fragment and its ability to interact with DNA binding proteins was observed, which is characteristic of non-specific binding (data not shown).

Secondly experiments with other protein extracts indicate that at the higher protein concentrations (10 μ g) the smaller DNA fragments also form DNA-protein complexes (data not shown). This could be a consequence of increasing the concentration of non-specific DNA binding proteins in the incubation mix and, therefore, increasing the probability of a non-specific DNA-protein complex forming with the smaller DNA fragments.

3.7.6 Competition experiments to determine if specific or non-specific interactions are occurring between the upstream region of *ANT1* and the maize nuclear protein extract.

To determine if the protein interactions with the DNA fragments were non-specific several competition experiments were carried out by adding an excess of unlabelled promoter fragment (*EcoRI* to *BamHI* which is between -2440 to -1530 bp upstream of the putative transcript initiation site) to the incubations. The rationale is that if a specific complex is formed with DNA from the promoter region the unlabelled specific competitor will bind the majority of the trans-acting protein. Consequently a significant reduction in the amount of end labelled promoter bound by a nuclear extract should be observed. No reduction would be expected if a non-specific DNA-protein complex is formed.

The specific competitor was a fragment from the 2.8 kb promoter containing A and C (*EcoRI* (-2440 bp)-*BamHI* (-1530) from pKG15, Figure 3.6A) which was subcloned into pUC19 (subclone pAC). The DNA-protein binding incubations were carried out as before (section 2.4.10) except linearised pUC19 was used as the non-specific competitor DNA rather than poly dIdC. Plasmid DNA has been shown to be less effective as a

competitor DNA than poly dIdC (data not shown) therefore the competition experiments were repeated using 1, 3 and 10 μg of plasmid as non-specific competitor.

The reason for using pUC19 as a non-specific competitor was to allow the substitution of pUC19 with pAC in the incubations requiring the addition of specific competitor. The quantity of competitor DNA in an incubation has been shown to be important (Campbell, 1984). If too little competitor DNA is present in an incubation the histone type proteins are not removed and will bind to the end labelled fragments. Conversely the addition of excessive competitor will result in the loss of specific DNA-protein interactions. If 3 μg of pAC is used in an incubation, as non-specific competitor, it will also contain the potential specific DNA binding sites in A and C, equivalent to the addition of 0.75 μg of purified EcoRI-BamHI promoter fragment (A+C Figure 3.6A). Thus the additional 0.75 μg of specific competitor does not have to be included in the incubation. The use of pUC19 and pAC means that the quantity of competitor DNA is equivalent in all the competition experiments.

When 1, 3 and 10 μg of pAC are substituted for pUC19 in an incubation mix the equivalent amount of the specific competitor added is 0.25, 0.75 and 2.3 μg . The quantity of end labelled promoter fragment is constant in each incubation (3.8 ng for the EcoRI-BamHI fragment). Therefore the ratio of 'unlabelled promoter fragment : end labelled promoter fragment' is either 66:1, 200:1 or 660:1 in the incubations (Figure 3.7A).

When pAC is added to the incubation the quantity of fragment A retained on the nitrocellulose did not decrease relative to the incubation which uses pUC19 as a competitor (Figure 3.7B). This would suggest that the DNA-protein complex formed with fragment A is due to a non-specific interaction. As the concentration of pUC19 or pAC is increased the amount of fragment A retained on the nitrocellulose decreases in a similar way for both plasmids. Increasing the competitor DNA will remove more of the non-specific binding proteins that remain in solution. When none of fragment A is retained by the nitrocellulose the non-specific binding can be assumed to be negligible. Under these conditions none of the DNA fragments from the promoter region are retained on the nitrocellulose. This result implies that no specific interactions could be

Figure 3.7

This figure presents the data from the competition experiments to determine if specific or non-specific interactions are occurring between the upstream region of *ANT1* and the maize nuclear extract. An identical protein-DNA interaction assay to the one described in figure 3.6 (section 2.4.10) was used for these experiments.

A The top table is a summary of the components of each incubation mix from I-VIII. The bottom table summarises the ratio of unlabelled A and C (specific competitor) DNA fragments compared to radio labelled A and C DNA fragments in each incubation mixture.

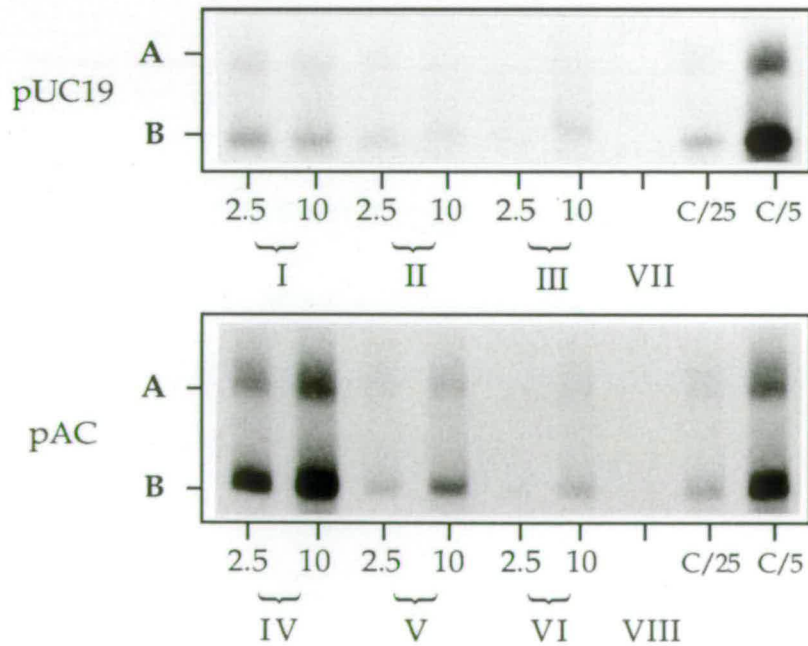
B The upper figure uses the plasmid pUC19 as competitor DNA whereas the lower figure uses the plasmid pAC as competitor DNA. The DNA fragments A and B are the same fragments identified to bind proteins in figure 3.6. For each of the incubations I-III and IV-VI both 2.5 and 10 μ g of nuclear protein extract were used which is indicated below the figure. The incubation mixes of VII and VIII did not include nuclear protein extract. The lanes marked either C/25 or C/5 represent a 25th or 5th respectively of the endlabeled DNA fragments from the *ANT1* putative promoter region used in each incubation mix.

A

INCUBATION	I	II	III	IV	V	VI	VII	VIII
pUC19 (μg)	1	3	10				1	
pAC (μg)				1	3	10		1
nuclear extract (μg)	2.5 or 10	2.5 or 10	2.5 or 10	2.5 or 10	2.5 or 10	2.5 or 10		
end labelled DNA (2.8 kb promoter)(ng)	11.8	11.8	11.8	11.8	11.8	11.8	11.8	11.8

amount of unlabelled AC (μg)				0.25	0.75	2.5		0.25
amount of end labelled AC (ng)	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
unlabelled : labelled AC AC				66	200	660		66

B



detected between DNA fragments containing the putative promoter region and the nuclear protein extracts.

3.8 SUMMARY

The quantity of competitor DNA included in the DNA nuclear protein incubation mixes appears to be critical. If the histone type proteins are not removed by the competitor DNA they will bind to the end labelled fragments as observed above. Conversely, the addition of excessive competitor will result in the loss of specific DNA-protein interactions (Campbell, 1984). A positive control is required to optimise this system for the detection of specific DNA-protein interactions in the *ANT1* promoter region. Ideally the positive control should be an enhancer or promoter element from a gene that is expressed in 3-day-old dark-grown maize seedlings.

A possible candidate for a positive control fragment is a promoter element upstream of a maize rRNA gene (Schmitz *et al.*, 1989). The expression of this gene has been demonstrated to be constitutive in all maize tissues. The promoter element, a gift from G. Feix, was subcloned as a 200 bp fragment. The conclusion from the limited data above is that no specific DNA-protein interactions occur in the *ANT1* promoter region from -2440 to +320 bp. The experiments with incubation conditions optimised using the positive control should allow the identification of specific DNA-protein interactions in the *ANT1* promoter region. These were not performed for the reasons that will be discussed in section 4.4

CHAPTER 4

CLONING A cDNA FOR *ANT2* AND 5' SEQUENCE ANALYSIS OF *ANT1* AND *ANT2*

4.1 RATIONALE

It was necessary to clone a full length cDNA for *ANT2* so that the 5' and 3' boundaries of the gene could be determined relative to the genomic sequence. Sequences from this clone also proved suitable as a specific probe for *ANT2* transcripts by northern blot analysis.

Prior to this work two distinct transcript initiation sites 5' to the *ANT* genes had been identified by primer extension analysis (Figure 3.2). The oligonucleotide used for these experiments was common to both *ANT1* and *ANT2* so it was not possible to determine if the initiation sites were common to both genes or whether they represented gene specific transcript initiation sites. The putative full length cDNA, pMANT1, shows 100% similarity with the genomic clone for *ANT1* with the 5' terminus corresponding to one of the transcript initiation sites which suggested that it may be specific for *ANT1* transcripts. By cloning the full length cDNA corresponding to *ANT2* I hoped to determine if the other transcript initiation site corresponded to the 5' end of this clone. Primer extension analysis with oligonucleotides specific for *ANT1* and *ANT2* was used to confirm the initiation sites.

The sequence similarity between the *ANT1* and *ANT2* is 94% and consequently there are no DNA probes that can be derived from the known sequence, which are suitable for investigating the differential expression of the two genes. Since the 3' untranslated regions from related cDNA clones are often more degenerate than the coding region, sequences derived from this region can be used as differential probes for highly similar genes. Recent studies have used probes derived from 3' untranslated regions to demonstrate differential expression of catalase genes in maize (Redinbaugh *et al.*, 1990) and tubulin genes in maize (Hussey *et al.*, 1990). If the two transcription initiation sites are specific for the two genes, discussed above, primer extension analysis on RNA from different tissues would also reveal if there is any differential expression (Chandler and Huiet, 1991).

4.2 SCREENING A MAIZE cDNA LIBRARY FOR ANT2

To isolate a full length clone for *ANT2* the gene specific oligonucleotide (Figure 3.2) was used to screen a cDNA library from 3-day-old dark-grown seedlings. The two advantages gained by using the specific oligonucleotide rather than a larger *ANT* DNA probe were:

- i) Only cDNA clones of *ANT2* would be isolated from the library
- ii) Near full length clones would be selected since the oligonucleotide is derived from a sequence at the 5' end of the *ANT* open reading frame (Figure 3.2).

The maize λ gt10 cDNA library was a gift from K. Palme (Cologne) and was constructed from polyA⁺ RNA isolated from 3-day-old dark-grown seedlings (MutinD-Fr7205034 hybrid seed). The library phage were grown and screened using standard techniques (Amersham protocol book *cDNA cloning system- λ gt10*) with the *Escherichia coli* strains NM514 and ED8654 (section 2.1.2) as hosts. For the first round 100,000 clones were screened and triplicate filters, cut from Hybond-N (Amersham), were lifted from each plate. Two filters were probed with end labelled specific oligonucleotides (section 2.4.4.3) while the third was probed with a DNA probe derived from pANT1 detailed in figure 3.1. Ten agar plugs were taken that contained putative *ANT2* clones as determined by hybridisation spots occurring on both the filters probed with the oligonucleotide. The third filter, probed with the *ANT* DNA probe, confirmed that all the clones were definitely *ANT* since the specific activity of the DNA probe was much greater than the oligonucleotide probe and consequently gave a much more intense hybridisation spot (data not shown). Other positive clones were also apparent that were not observed on the filters probed with the oligonucleotide. These additional positives presumably represented *ANT1* clones and short *ANT2* clones.

A further three rounds of screening using the oligonucleotide probe were carried out and four clones were purified to homogeneity. DNA from the phage was isolated and the insert sizes were observed on an agarose gel after digestion with EcoR1 to release the insert from the lambda arms. The largest insert, approximately 1.6 kb, was the same size as pMANT1 (section 3.2.1) and therefore assumed to be a near full length clone. The 1.6 kb

insert was subcloned into a pBluescript II KS- cloning vector and sequenced using -40 primer, reverse primer and specific oligonucleotides complementary to appropriate sequences in the clone (section 2.4.7.1).

4.3 SEQUENCE ANALYSIS OF pMANT2, A cDNA CLONE OF ANT2

4.3.1 Confirmation that the cDNA clone pMANT2 has sequence identity with ANT2

Sequencing of the cDNA revealed the insert size to be 1511 bp. Analysis of the sequence using the GAP computer program (section 2.5) revealed 57 bp at the 5' end containing no similarity with *ANT2* (discussed in section 4.3.5). The rest of the sequence was highly similar with only 26 differences. As the genomic library and cDNA library were derived from different maize lines (section 2.1.6 and 2.1.7) the differences could be interpreted as mutations that had occurred since the segregation of the genotypes. Consistent with this hypothesis, the differences are more common in the 5' untranslated and 3' untranslated regions which would be expected as these regions are usually less conserved relative to the open reading frame. The differences in the open reading frames do not affect the predicted amino acid sequence. This cDNA clone is thought to correspond to *ANT2* as it has the same predicted amino acid sequence and restriction endonuclease sites characteristic of *ANT2*. Therefore this cDNA clone was designated pMANT2 (Figure 4.1).

4.3.2 Differences between pMANT2 and ANT2 correlate with sequencing errors in ANT2

The 26 differences between *ANT2* and pMANT2 are shown in Figure 4.2B. It is striking that most of the differences improve the similarity between the two cDNA clones pMANT1 and pMANT2. The 5' untranslated region from the genomic clone of *ANT2* was resequenced to check that the differences were real. Nine of the differences were within the region resequenced and all were sequencing errors of the *ANT2* clone. The other 17 differences located within the open reading frame and in the 3' untranslated region have not been checked by resequencing the *ANT2* clone. A number of these differences may also be due to sequencing errors of the *ANT2* clone. Since the genomic and cDNA libraries were

Figure 4.1

Comparison of the *ANT* cDNA clones pMANT1 (upper sequence) and pMANT2 (lower sequence). The predicted amino acid sequence for the pMANT1 clone is above its nucleotide sequence. The predicted amino acid sequence for pMANT2 is below its nucleotide sequence where different from pMANT1.

The similarity between the nucleotide sequences in the coding region is 94% while the 3' untranslated region has a similarity of 88%. Portions of the 3' untranslated region (77% similar) from the marked *Sau3A* restriction endonuclease site to the 3' terminus in both cDNA clones were used as differentially specific probes for northern blot analysis. Similarly the 5' terminus sequences from the 5' end to the *StyI* restriction endonuclease sites were subcloned for use as differentially specific probes. A general *ANT* specific RNA probe (universal *ANT* probe) was complementary to the pMANT2 sequence from the marked *PstI* site to the 3' terminus and had 91% similarity to the equivalent pMANT1 sequence.

The consensus sequence for the translation start site (Joshi, 1987a) is shown under two ATG codons. The first ATG codon represents the start of the extended open reading frame identified by Sarah (1991) and in this thesis (see section 4.3.3). The boxed ATG represents the translational start site incorrectly identified by Bathgate *et al.*, (1989). The boxed sequence in the 3' untranslated region represents a putative polyadenylation signal (Joshi, 1987b).

The symbols indicate:



= site of intron B



= amino acid processing site



= 3' polyadenylation site of the cDNA clone pANT-1

Styl

M A D

1 CGCGCCTCTGAAASTTCGGAGCCGCCGCCGCTCCGCTCCC...TCGCCCTCCCGTCCCA...AGCCCAATAGTTTCAAGTGGCAATGGCGGAC 93
 1GTGAGGGCCCGCCAGACCGCTCCCTTCCCGCCTCCGGTGC...CCAAGGTCAGGCCAACAGTTTGAAGTGGTAAATGGCGGAC 86

CAATGGCT

Q A N Q P T V L H K L G G Q F H L R S I I S E G V R A R N I C P S
 94 CAGGCTAACCAACCCTGCTTCATAAGCTCGGTGGCCAGTCCACCTGCGCTGCATCTCTGAAGGTSTACGGGCCGTAACATATGCCCATCTG 193
 87 CAGGCTAACCAACCCTGCTTCATAAGCTAGGTGGCCAGTCCACCTGAGCTCCAGCTTCTCTGAAGGTSTACGGGCCGTAACATATGCCCATCTG 186

S S F

V S S Y E R R F A T R N Y M T Q S L W G P S M S V S G G I N V P V M
 194 TCTCATCTTATGAAAGGAGATTGCCACAAGGAACATGACCCAGAGCCTTGGGGCCCTCAATCTCTGTAGCGGTGGCATCAATGTCCAGTGAT 293
 187 TCTCACCTTATGAAAGGAGATTGCCACGAGGAACATGACCCAGAGCCTTGGGGCCCTCAATCTCTGTAGCGGTGGCATCAATGTCCAGTGAT 286
 F P

CAATGGCT

↓

O T P L C A N A P A E K G G K N F M I D F M M G G V S A A V S K T
 294 GCAGACCCCGCTCTGTGCTAATGCTCCTGCTGAGAAAGGAGGCAAGAACTTCATGATTGATTTCATGATGGCGGTGTTTCAGCTGCTGTTTCAAGACT 393
 287 GCCGACTCCGCTTTTGGCAATGCTCCAGCCGAGAAAGGTGGCAAAACTTCATGATTGATTTCATGATGGCGGTGTTTCAGCTGCTGTTTCAAGACT 386
 P F

A A A P I E R V K L L I O N O D E M I K S G R L S E P Y K G I V D
 394 GCTGCTGCTCCATCGAGCGTGTGAAGCTGCTTATTGAGAACCAAGATGAGATGATTAACTCTGGTAGGCTATCAGAGCCGTACAAGGTTATTGTTGACT 493
 387 GCAGTCTGCTCCATTGAAGGAGTGAAGCTGCTTATTGAGAACCAAGATGAGATGATTAACTCTGGTAGGCTATCAGAGCCGTACAAGGTTATTGTTGACT 486
 PstI

C F K R T I K D E G F S S L W R G N T A N V I R Y F P T Q A L N F A
 494 GCTTCAAACGTACCATTAAGGATGAAGGTTTCTCTTCTTGTGGAGGGTAACTGCTAAATGTTATTCGTTACTTCCCTACTCAGGCTTTGAACCTTTGC 593
 487 GCTTCAAACGTACCATTAAGGATGAAGGTTTCTCTTCTTGTGGAGGGTAACTGCTAAATGTTATTCGTTACTTCCCTACTCAGGCTTTGAACCTTTGC 586

F K D Y F K R L F N F K K D R D G Y W K W F A G N L A S G G A A G
 594 ATTTAAGGACTACTTCAAGAGSTTGTCAACTCAAGAAGGATAGGGATGGCTATTGGAAGTGGTTTGTGGCAACCTGGCCTCTGGTGGTCTGCTGGT 693
 587 GTTTAAGGACTACTTCAAGAGSTTGTCAACTCAAGAAGGATAGGGATGGTACTGGAAGTGGTTTGTGGCAACCTGGCCTCTGGTGGTCTGCTGGT 686

A S S L F F V Y S L D Y A R T R L A N D A K A A K G G G E R Q F N
 694 GCTTCCTCTTTGTTTTGTGTACTCCCTGACTACCGGAGAACAAGGTTGGCTAATGACCGCAAGGCTGCCAAGGGAGGAGGTGAAGGCAAGTCAATG 793
 687 GCTTCCTCTTTGTTTTGTGTACTCCCTGGATTATGCGAGGCAAGGTTGGCCAAATGATGCCAAGGCTGCCAAGGGAGGAGGCGATAGACAATCAATG 786

D

G L V D V Y R K T L K S D G I A G L Y R G F N I S C V G I I V Y R G
 794 GGCTTGTGATGCTACCGCAAGACACTCAAGTCTGATGGTATTGCTGGGCTTACCCTGGATTAACTATCTCCTGTGTTGGAATCATTGTTTATCGTGG 893
 787 GTCCTGTGGATGCTACCGCAAGACACTCAATCTGATGGTATTGCTGGGCTTACCCTGGATTAACTATCTCCTGTGTTGGAATCATTGTTTATCGTGG 886

L Y F G L Y D S I K P V V L T G N L Q D N F F A S F A L G W L I T
 894 TCTGTACTTTGGGCTCTATGATCCATCAAGCCAGTGTGCTTACTGGCAACCTCCAGGACAATTTCTTTGCCAGTTTCGCTCTGGGTTGGCTGATCACT 993
 887 CCTGTATTTCCGACTTACGATTCTATCAAGCCAGTGTGCTTACTGGCAGCCTCCAGGACAACCTTTTCCAGTTTGTCTGGGTTGGCTGATCACT 986

S

N G A G L A S Y P I D T V R R R M M M T S G E A V K Y K S S L D A
 994 AATGGTGTGGTCTTGCATCTTACCCCATCGATACCGTCCGAGAAGGATGATGATGACATCTGGTGAGGCTGCAAGTACAAGAGCTCCTTGGACCGGT 1093
 987 AACGGTGTGGTCTTGCATCTTACCCCATGATACTGCTCCGAGAAGGATGATGATGACTTCTGGTGAGGCTGCAAGTACAAGAGCTCATTGACCGCAT 1086

F Q Q I L K K E G P K S L F K G A G A N I L R A I A G A G V L S G Y
 1094 TCCAGCAGATTCTAAGAAGGAAGGCCCAAGTCCCTGTTCAAGGGTGTGGTGAATCTTCTCGTGCCATTGCTGGTGTGCTTCTGGCTA 1193
 1087 TCCAGCAGATCTTAAAGAAGGAAGGCCCAAGTCCCTGTTCAAGGGTGTGGTGAATCTTCTCGTGCCATTGCTGGCGCTGGTGTGCTTCTGGCTA 1186

D Q L Q I L F F G K K Y G S G G A *
 1194 TGACCAGCTCCAGATCCTCTTCTCGGAAAGAAGTACGGCTCCGGCGGTGCTTAAATGGAGAAAATGATGACGAACAAGAGCAGTGTGTTCCCGGTC 1293
 1187 CGACCAGCTCCAGATCCTCTTCTCGGAAAGAAGTACGGCTCCGGCGGTGCTTAAATGGAGAAAATGATGACGAACAAGAGCA...GTCGCTCCCGGTC 1284

CTTTCCAATCAGGATCTGGTGAAGTTTTCGCTTTCATATCGAAGAAATTAATAATTCATGTAGAGGAGGATTCTTCCAACAATTTGTTTGGATGGAG 1393
 1285 CAGCCAGTCAGGATCTGGTCAAGTTTTCGCTTTCATATCGAAGAAATTAATAATTTATGTA...GGAGAATTTTCCAAC.ATTCGTTTTGGCGGAG 1380

Sau3A

▽

ACTTAGATCTCAAACACTCGTAACATCTACTAGGTGCTGACATCGGTCCCAAAA...ACATTTATGCTCATAGACTCTGCCCTTCGTTTGTGACT 1489
 1381 ACATTAGATCTCAAACACTCGTAACATCTACTAGGTGCTGACATCGGTCCCAAAAACATGGTCATAGGCTGTCTAGTCTGCCCTTCGTT...GCAGC 1478

CGGGCAATTTTCTTGCATACAATGCCTTCGTTAAGTATCGGATGAGTGTGCGACTAATTATTGTTGCTTTACCTTTTGCAAAA 1579
 1479 CGGGCAATTTTCTTACATATATTGCCTTTAAT 1511

constructed from different maize lines it cannot be discounted that some of the differences are due to mutations.

Resequencing of the 5' untranslated region from pMANT1 and the genomic clone of *ANT1* also revealed sequencing errors in the original sequence data (Sarah, 1991). A comparison of pMANT1 and *ANT1* reveals a further 12 differences in the open reading frame and the 3' untranslated region which are shown in Figure 4.2A. Most of the differences improve the similarity between the two cDNA clones which was also the case for pMANT2 and *ANT2*. As discussed above, this suggests that most of the differences are due to sequencing errors in the genomic sequence. The *ANT1* genomic clone has not been resequenced to check this.

4.3.3 A larger open reading frame for the ANT protein

Correction of the sequencing errors in the 5' untranslated region of pMANT1, *ANT1* and *ANT2* has a dramatic effect on the interpretation of the *ANT* gene structure. An ATG 174 nucleotides 5' of the ATG originally postulated as the start of translation (Bathgate *et al.*, 1989) is now in frame with the open reading frame and increases the size of the predicted protein by an additional 58 amino acids (Figure 4.1). The sequence surrounding the new ATG also fits the consensus sequence for plant genes proposed by Joshi (1987a). An enigma associated with the ANT protein has been resolved with the discovery of the extended open reading frame.

A specific processing event occurs when the ANT protein is imported into mitochondria (Purdue, 1988; Sarah, 1991; Winning *et al.*, 1992). The processing results in the loss of 7.5 kDa from the amino terminus and a concomitant reduction in size of the ANT protein from 38 kDa to 30.5 kDa. Using the original ATG there are only 19 amino acids N-terminal to the processing site as opposed to 77 amino acids from the new in frame ATG (Figure 4.1). Assuming the average mass of an amino acid is 110 Da, 77 amino acids equates favourably with 7.5 kDa estimated as the size of the N-terminal presequence.

Figure 4.2

A This figure shows the 12 differences between pMANT1 and the *ANT1* genomic clone. The nucleotides which are different between the cDNA and genomic sequences are in 'bold' type. The number corresponds to the position of each difference with respect to the cDNA clone pMANT1. The type of difference from the genomic clone to the cDNA clone is indicated by a letter; C = change of nucleotide, I = an insertion of a nucleotide, S = two nucleotides have been swapped and D = the deletion of a nucleotide. The number associated with each letter indicates; 1 = the difference does not make pMANT1 similar to pMANT2 but *ANT1* is similar to pMANT2, 2 = the difference does not make pMANT1 similar to pMANT2, 3 = the difference makes pMANT1 similar to pMANT2.

B This figure shows 26 differences between pMANT2 and the *ANT2* genomic clone. The first nine of these differences have been identified as sequencing errors in the *ANT2* genomic clone (see section 4.5.2). The number corresponds to the position of each difference with respect to the cDNA clone pMANT2. The type of difference from the genomic clone to the cDNA clone is indicated by a letter as above. The number associated with each letter indicates; 1 = the difference does not make pMANT2 similar to pMANT1 but *ANT2* is similar to pMANT1, 2 = the difference does not make pMANT2 similar to pMANT1, 3 = the difference makes pMANT2 similar to pMANT1.

Both pMANT1 and pMANT2 have been sequenced on both strands and these sequences are thought to be errorless (Winning *et al.*, 1991). Sequence of the *ANT1* and *ANT2* genomic clones has previously been found to have several sequencing errors (see section 4.5.2). Consequently the mismatches between the *ANT* cDNA and genomic clones may be due to further sequencing errors in the genomic clones. Alternatively, since the cDNA and genomic libraries were constructed from different maize lines the mismatches could be due to line differences between the cDNA and genomic clones. Type-1 and type-2 mismatches may be due to line differences or sequencing errors. Type-3 differences are probably due to sequencing errors in the genomic sequences. It appears that most of the differences between the cDNA clones and the genomic clones are due to sequencing errors in the genomic clone sequence.

A DIFFERENCES BETWEEN pMANT1 AND ANT1

ANT1	GGTGG	TCGGAG	GTGCA	AAATC	GGACT
pMANT1	331-GGAGG	385-TCAAAG	474-GTACA	823-AAGTC	904-GGGCT
pMANT2	GGTGG	TCGAAG	GTACA	AAATC	GGACT
	C1	C1 C3	C3	C1	C1
	ATAAT	TGTAGA	ATTTC	AG TG TG	
	1257-AT AT	1260-TGATGA	1330-ATATC	1428-AGGTGCTG	
	AA AT	TGATGA	ATATC	AGGTGGTG	
	D3	S3 S3	C3	I3 I3	

B DIFFERENCES BETWEEN pMANT2 AND ANT2

ANT2	CTGGG	GTCCACCTG	CCGACT	TTGGAA		
pMANT2	117-CTAGG	128-GTCCACCTG	142-CCAGCT	148-TT CAA		
pMANT1	CTCGG	GTCCACCTG	CGATCA	TT CAA		
	C2	C3 S3 S3 C3	S3 S2	D3 C3		
	GGAAT	GCGGA	CAAGA	GGCTATTG	TCATCC	CGAGG
	267-GGCAT	315-GCCGA	429-CAGGA	636-GGTTACTG	799-TCTACC	882-CGTGG
	GGCAT	GCTGA	CAAGA	GGCTATTG	TCTACC	CGTGG
	C3	C2	C1	C1 C1	S3 S3	C3
	GGGTG	CT CC	TT ATAT GA	CTCCAAAACA TC	AATCT	
	1055-GGCTG	1276-CTCCC	1318-TTCATATCGA	1389-CT CAAAACACTC	1402-AA CT	
	GGCTG	TTCCC	TTCATATCGA	CT CAAAACACTC	AA CT	
	C3	I3	I3 I3	D3 I3	D3	
	GGCGAT GGT					
	1422-GGC ATGGGT					
	GAC ATCGGT					
	D3 I3					

4.3.4 Analysis of the 3' untranslated sequence of the maize ANT genes

Comparison of the 3' untranslated sequence of *ANT1* and *ANT2* reveals that the cDNA clones contain different polyadenylation sites (Figure 4.1). This has also been shown to be true for the *ANT* cDNA clones isolated from potato (Winning, pers. comm.) and is a common feature of other plant genes (Dean *et al.*, 1986; Sachs *et al.*, 1986). A sequence containing a putative polyadenylation signal was found in both genes with a 1 bp substitution compared to the animal consensus sequence 5'-AATAAA-3'. This is not unusual since a detailed analysis of plant polyadenylation signals has revealed that 25 out of 46 plant genes have a 1 bp substitution from the animal consensus (Joshi, 1987b).

The 3' untranslated region is highly conserved between pMANT1 and pMANT2 (88%) but a region from a *Sau3AI* restriction site to the end of the pMANT2 clone is sufficiently different (77%) to use as a differential probe. Its use for this purpose will be discussed in more detail in section 5.4.1.3.

4.3.5 The 5' termini of the *ANT* cDNA clones are similar despite no similarity between the cDNA clones and the *ANT* genomic sequence

Comparison of the pMANT2 with *ANT2* sequences revealed 98% similarity except for the first 57 bp which has no similarity to the *ANT2* genomic sequence (data not shown). When pMANT1 and pMANT2 are compared the first 64 bp of pMANT1 are highly similar to the first 57 bp of pMANT2 (Figure 4.1) which suggests that the additional bases at the 5' end of pMANT1 are not artifacts as first postulated (section 3.2.2). There are two hypotheses for the origin of these unidentified sequences which were investigated as described in section 4.6.

The first hypothesis is that they are exons in the 5' untranslated region of the *ANT* genes. The known genomic sequence extends 463 bp (*ANT1*) and 570 bp (*ANT2*) 5' of the initiation ATGs and has no similarity with the 5' bases (64 bp of pMANT1 and 57 bp of pMANT2) from the two cDNA sequences. If the cDNA 64 and 57 bp sequences do represent 5' exons the introns must be greater than 442 and 549 bp for *ANT1* and *ANT2*

respectively (these sizes represent the sequenced genomic sequences 5' of the breakdown in similarity between the cDNA and genomic clones). If this hypothesis is correct either the transcription initiation sites and promoter sequences have been incorrectly identified (section 3.2.2) or there are two promoters. The genes for the zein storage proteins in maize have two promoters which direct transcription from two initiation sites separated by 1 kb (Langridge and Feix, 1983).

The second hypothesis is that a trans-splicing event may occur with the addition of an extra 60-70 bp to the *ANT* transcripts. The first example of trans-splicing was described in the primitive eukaryote *Trypanosoma* (Van der Ploeg *et al.*, 1982) and both mitochondria and chloroplast organelles commonly use trans-splicing (Wissinger *et al.*, 1991, Koller *et al.*, 1987). The first complex organism discovered to use a trans-splicing mechanism was the nematode *Caenorhabditis elegans* (Graham *et al.*, 1988). The actin and ubiquitin genes are both processed using trans- and cis-splicing mechanisms. *Euglena gracilis* is another organism that possesses trans- and cis-splicing within the same nuclear gene transcripts (Tessier *et al.*, 1991). *Euglena* and nematodes are evolutionarily distant and, hence, trans-splicing could potentially be an essential mechanism which has been conserved in higher eukaryotes.

A small amount of trans-splicing has been observed *in vitro* between sites that preferentially undergo cis-splicing (Padgett *et al.*, 1986). It is postulated that trans-splicing can potentially occur between transcripts with conserved splice sites but it is avoided *in vivo* by the active separation of the nascent RNA transcripts before splicing. Although trans-splicing has not been reported in higher eukaryotes it may occur.

4.4 A SUMMARY OF THE GENE STRUCTURE OF ANT WHICH IS MORE COMPLICATED THAN PREVIOUSLY CONCLUDED

The gene structure which had previously been described for *ANT* by Bathgate *et al.* (1989) is summarised in section 3.2 and figure 3.1. The assumption that the promoter region was within the *ANT1* and *ANT2*, genomic clones (Bathgate *et al.* (1989), formed the basis for many of the experiments carried out to achieve the objectives of this thesis. These include the DNA-protein interaction experiments described in section 3.7, the construction of translational fusions in section 3.5, the construction of

transgenic plants with the pG2-GUS translational fusion clone (data not shown) and primer extension analysis using *ANT1* and *ANT2* specific oligonucleotide primers discussed in section 4.1.

I have now shown that the 5' gene structure of *ANT* is more complicated than previously concluded. This was identified because many of the experimental results I obtained were unusual and could not be interpreted unless the gene structure described by Bathgate *et al.* (1989) was incorrect. These included the inability to observe specific interactions between DNA fragments containing the putative promoter region and the nuclear protein extracts. In addition the primer extension products reported by Bathgate *et al.* (1989) were not observed using *ANT1* and *ANT2* specific oligonucleotide primers (figure 3.2). For both oligonucleotide primers faint bands correlating to initiation sites further upstream were observed (data not shown). Finally the cloning and sequence analysis of an *ANT2* cDNA clone, pMANT2, was observed to have a sequence at its 5' terminus which was similar to an equivalent sequence in the *ANT1* cDNA clone, pMANT1 (section 4.3.5). Until the similarity between the pMANT1 and pMANT2 5' termini had been identified the pMANT1 5' terminus was considered to be the result of a cloning artifact (section 3.2.2).

The experiments described in the remainder of this chapter were performed to characterise the 5' gene structure of *ANT1* and *ANT2*. The gene structure of *ANT* described by the following experiments are briefly listed below and summarised in Figure 4.3.

- i) Both *ANT* genes contain multiple transcription initiation sites, but the position and number of sites are not conserved between the two genes (section 4.8.1).
- ii) The sizes of the 5' UTR's are between 124 bp and 356 bp as predicted from the various primer extension products (section 4.8.1).
- iii) Both *ANT* genes have a minimum of two untranslated exons and two large introns have been identified which separate the untranslated exons (intron A and intron B). The 5' and 3' splice sites of intron A have not been accurately determined for *ANT1* or *ANT2* but the intron size is in excess of 2.4 kb for *ANT2*, since sequence similar to the first exon, as determined by RNA sequencing, is not

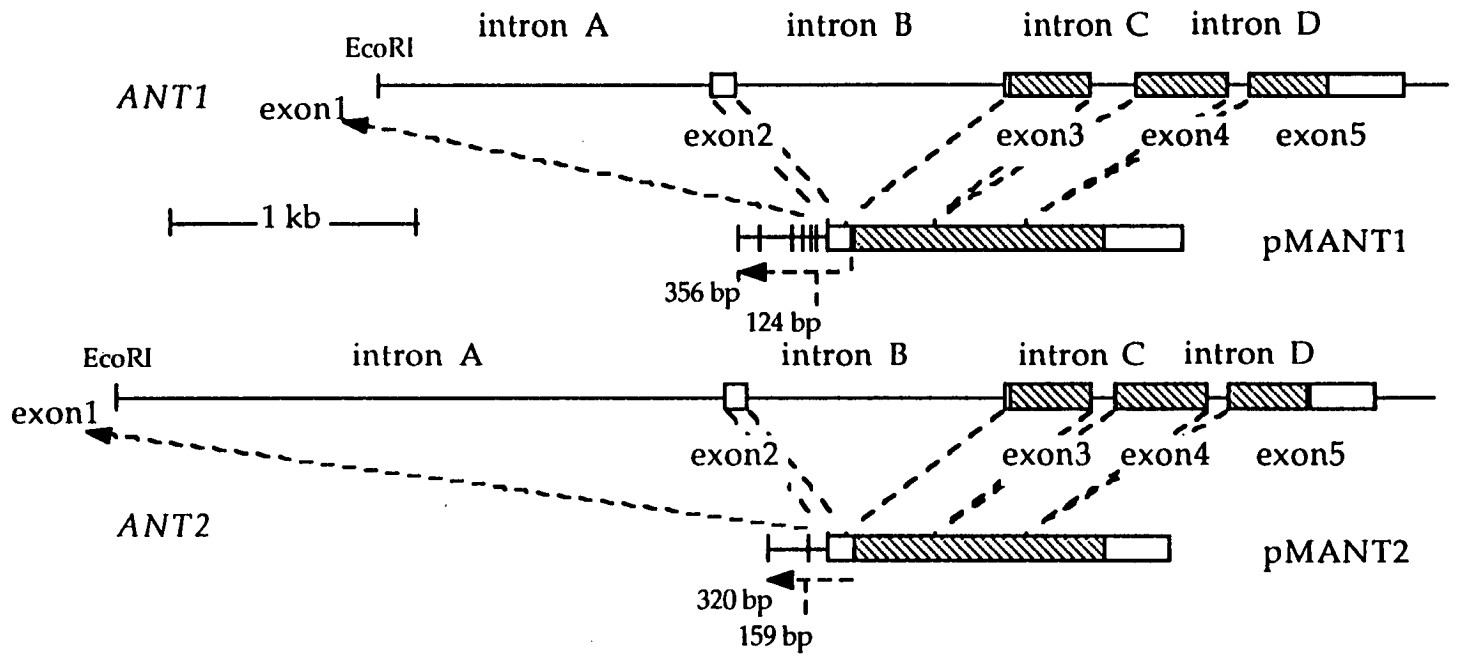
Figure 4.3

A summary of the ANT gene structure with the sizes of intron, exon and UTRs shown in the table below .

	<i>ANT1</i> bp	<i>ANT2</i> bp
exon 1	104-336	139-300
2	approx 85	approx 78
3	341	341
4	372	372
5	pANT-1 476 pMANT1 631	pMANT2 570
intron A	>1.3 kb	>2.4 kb
B	1063	1021
C	174	94
D	80	81
5'-UTR	124-356	159-320
3'-UTR	pANT-1 179 pMANT1 334	pMANT2 273

For the genomic clones the hatched boxes denote the coding sequence. The open boxes denote the untranslated sequence. The lines denote intron sequence except at the 3' end of the untranslated region where it denotes a continuation of the genomic sequence for 4030 bp in *ANT1* and 1360 bp in *ANT2*.

For the cDNA clones pMANT1 and pMANT2 the hatched boxes denote the coding sequence. The open boxes denote the untranslated sequence present in the clone. The lines at the 5' end represent the untranslated sequence which is not part of the clone but has been shown to exist by primer extension. The vertical lines denote the various transcript initiation sites.



present in the 5' region of the genomic clone (section 4.8.3). Intron B is 1063 bp and 1021 bp in size for *ANT1* and *ANT2* respectively and is separated from intron A by exon 2 which is approximately 85 and 78 bp for *ANT1* and *ANT2* respectively (section 4.6 and 4.8.2). The start of translation is in exon 3 and is 21 bp 3' of the intron B sequence (section 4.6).

iv) The genomic sequences corresponding to the promoter region and exon 1 of the two *ANT* genes have not been identified and are presumed to be 5' of the cloned genomic DNA (section 4.8.3).

v) In summary, the *ANT* genes have at least 5 exons termed exon 1 to exon 5 and at least 4 introns termed intron A to intron D (Figure 4.3).

4.5 SOUTHERN BLOT ANALYSIS OF THE *ANT* GENOMIC CLONES WITH THE 64 bp AND 57 bp 5' TERMINI OF pMANT1 AND pMANT2 TO IDENTIFY THE LOCATION OF THE PUTATIVE 5' EXONS (EXON 2)





To test the hypothesis that the 5' termini are exons in the upstream region of *ANT1* and *ANT2*, the genomic clones were probed with specific RNA probes (section 5.4.1.2) corresponding to the 5' termini of pMANT1 and pMANT2. The presence of a *StyI* restriction enzyme site that coincides exactly with the point of sequence divergence between the cDNA and genomic clones was convenient for sub cloning the 5' termini of pMANT1 and pMANT2 (Figure 4.4). The advantage of subcloning these small fragments was that specific RNA and DNA probes made for the 5' termini will not be contaminated with other *ANT* transcript sequences.

For *ANT1* approximately 2 kb of the cloned 5' region was unsequenced while for *ANT2* the unsequenced 5' region was 3 kb (Figure 3.1). The 5' termini from pMANT1 and pMANT2, if they were 5' exons, would probably occur in the unsequenced regions of the genomic clones.

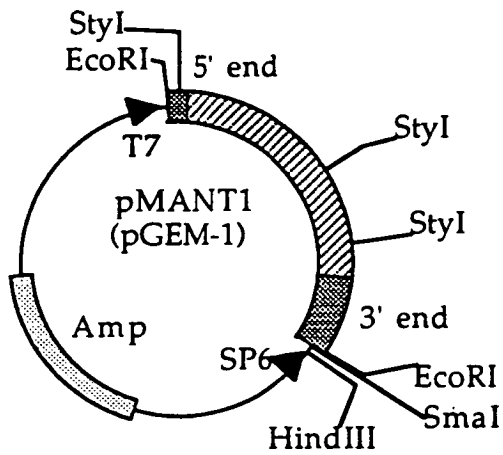
Both the 5' genomic-*GUS* fusion clones, pG1-*GUS* and pG2-*GUS* (section 3.5), were digested with restriction enzymes which cut progressively larger sequences from the 5' region of each gene (Figure 4.5A). The digested plasmid DNA was separated by electrophoresis through an agarose gel and

Figure 4.4

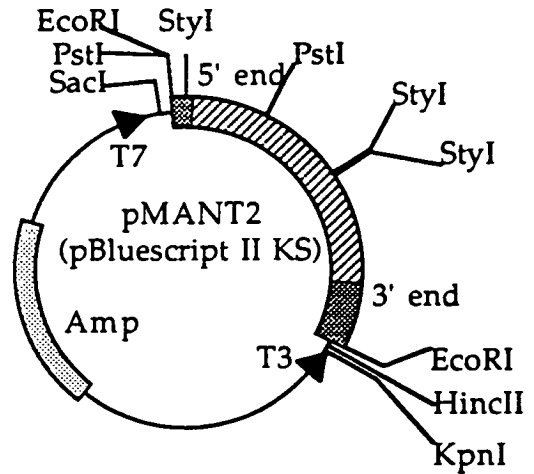
The upper two clones are a map of the pMANT1 and pMANT2 cDNA clones. The various symbols are:

-  = 3' or 5' untranslated sequence of *ANT*
-  = coding sequence of *ANT*
-  = Amp resistance gene
-  = T3 or T7 promoter sequence

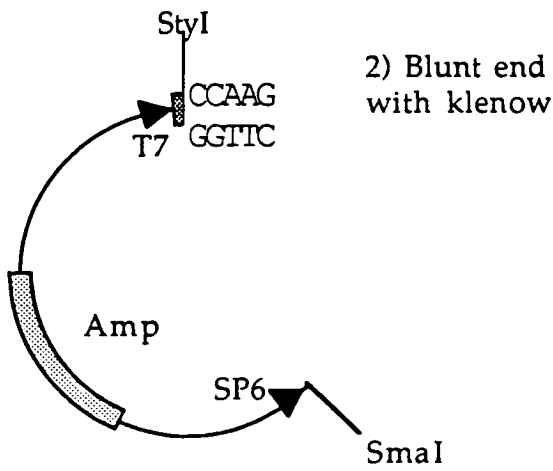
The scheme shows the cloning strategy for the 5' termini of pMANT1 (first 64 bp) and pMANT2 (first 51 bp). The subclones were termed pG1-64 and pG2-51.



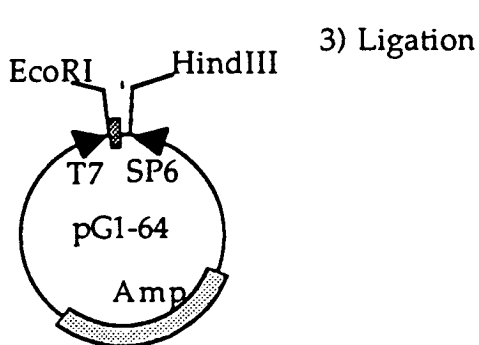
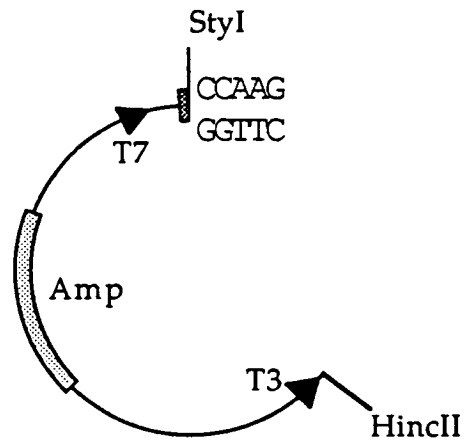
1a) Digest with StyI and SmaI



1b) Digest with StyI and HincII



2) Blunt end with klenow



3) Ligation

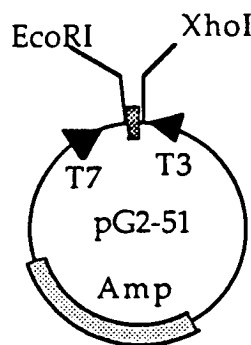






Figure 4.5

A A diagrammatic representation of the fragments which result from digesting pG1-GUS and pG2-GUS with various restriction endonuclease enzymes. The restriction endonuclease enzymes used in each case are listed to the left of the fragments. The numbers beneath some of the fragments are approximate sizes in kb to the nearest 0.1 kb. The SphI fragments had been isolated for subcloning (section 4.6) and were subsequently used in this experiment.

The symbols used in this figure are summarised below:

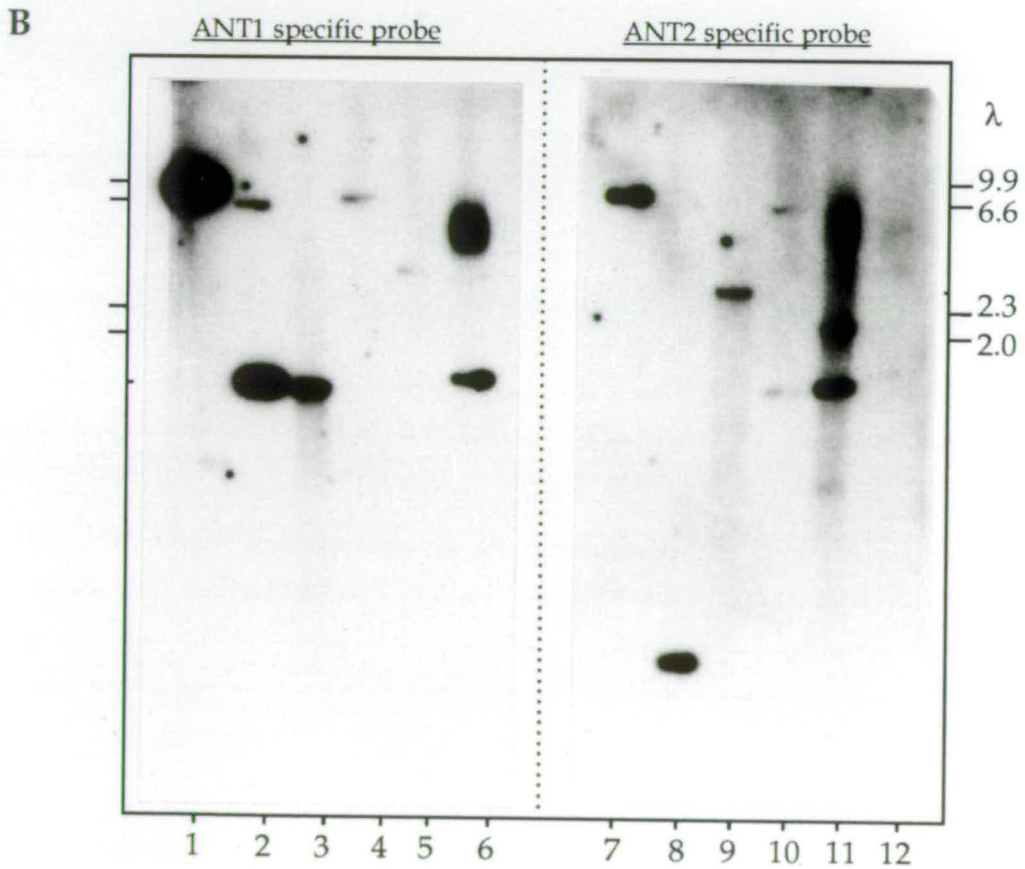
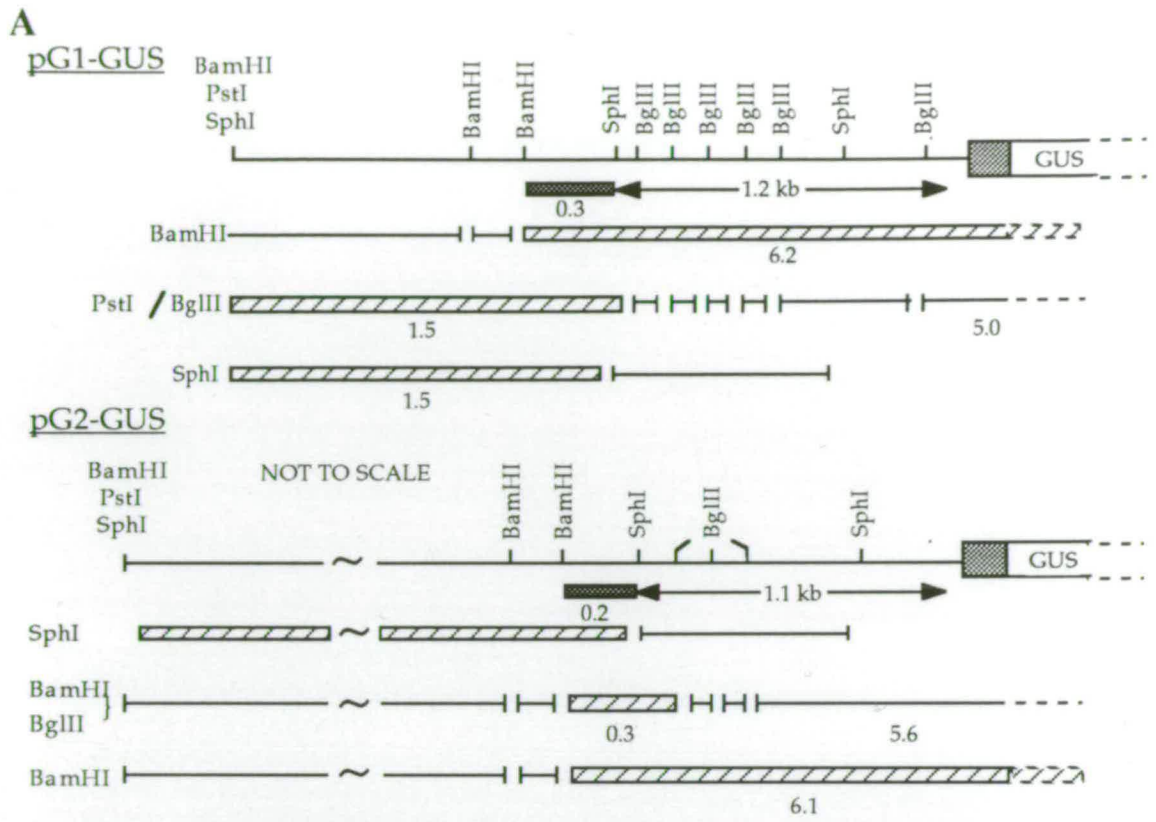
-  = coding sequence of *ANT*
-  = the fragment from the digested genomic DNA which hybridises to the cDNA 5' terminus probe
-  = region common to all the fragments which hybridise to the cDNA 5' terminus probe
-  = putative transcript initiation site and point of divergence between the cDNA and genomic clones

B A Southern blot of the DNA fragments which result from digesting pG1-GUS and pG2-GUS with various restriction endonuclease enzymes.

Lanes 1-6 were probed with an RNA probe of the *ANT1* specific 5' terminus clone pG1-64.

Lanes 7-12 were probed with an RNA probe of the *ANT2* specific 5' terminus clone pG2-51. The DNA in each lane was:

Lane 1	pG1-GUS digested with BamHI
Lane 2 and 10	pG1-GUS digested with BglII /PstI
Lane 3	SphI fragments isolated from pG1-GUS
Lane 4 and 8	pG2-GUS digested with BglII/BamHI
Lane 5 and 11	pMANT1 digested with EcoRI
Lane 6 and 12	pMANT2 digested with EcoRI
Lane 7	pG2-GUS digested with BamHI
Lane 9	SphI fragments isolated from pG2-GUS



Southern blotted (section 2.4.5.1). The immobilised DNA was then probed with the specific RNA probes.

Both of the specific RNA probes hybridised to the plasmid DNA of the genomic subclones thus confirming the presence of exons in the 5' unsequenced region of the genomic clones (Figure 4.5B). The hybridisation of the high molecular weight fragment after the exon has been released by a restriction enzyme digest is due to slight plasmid contamination in the probe (Figure 4.5B).

The position of the exons (exon 2) were estimated by determining which restriction enzyme released the exon sequence from the plasmid. The *ANT1* exon can be localised between 1.2 kb and 1.5 kb 5' of the point of divergence between the pMANT1 clone and the *ANT1* genomic clone. The *ANT2* exon can be localised between 1.1 kb and 1.3 kb 5' of the point of divergence between the pMANT2 clone and the *ANT2* genomic clone (Figure 4.5A+B). This Southern blot analysis also demonstrated that the 5' sequence probes derived from pMANT1 and pMANT2 are specific for *ANT1* and *ANT2* sequence (section 5.4.1.2.).

4.6 SEQUENCING THE 5' REGION OF BOTH *ANT1* AND *ANT2* GENOMIC CLONES

The unsequenced 5' regions (section 4.5) in the *ANT* genomic clones were sequenced to determine the exact location of the 5' exons and obtain sequence data to identify the *ANT* promoter consensus sequences.

The sequencing strategy was to subclone the unsequenced 5' regions of *ANT1* and *ANT2*, as two *SphI* fragments from pG1-GUS and pG2-GUS, into the pBS+ cloning vector (section 2.1.4)(Figure 4.6). The pBS+ cloning vector has specific sequences 5' and 3' of the multiple cloning site polylinker corresponding to the -40 and reverse sequencing primers (Figure 4.6). The Southern blot data above suggests the 'small *SphI* fragment' subclones (pSS1 and pSS2) should contain intron (intron B) sequence while the exons were expected 5' of the *SphI* site in the 'large *SphI* fragment' subclones (pLS1 and pLS2)(Figure 4.6).

The pLS1 and pLS2 clones contained the putative promoter region (now known to be intron A) and could not be completely sequenced using the

Figure 4.6






A This restriction map of the pG2-GUS clone (3.9 kb of genomic sequence upstream of *ANT2* fused to the GUS reporter gene) indicates the SphI fragments which were cloned into the pBS+ cloning vector. These clones were termed pLS2 (Large SphI fragment of *ANT2*) and pSS2 (Small SphI fragment of *ANT2*) and were used as templates to sequence the genomic DNA using the -40 and reverse (Rev) sequencing primers. The arrows indicate the sequencing strategy. The nested deletions above the restriction map were created using XhoIII by Dr J Ross and were used to sequence the internal region of the large SphI fragment.

RNA (antisense) probes were generated from the pLS2 clone, which had been digested with BamHI, using the T3 promoter in the pBS+ cloning vector. These RNA probes were used for RNase protection analysis (see section 4.8.2)

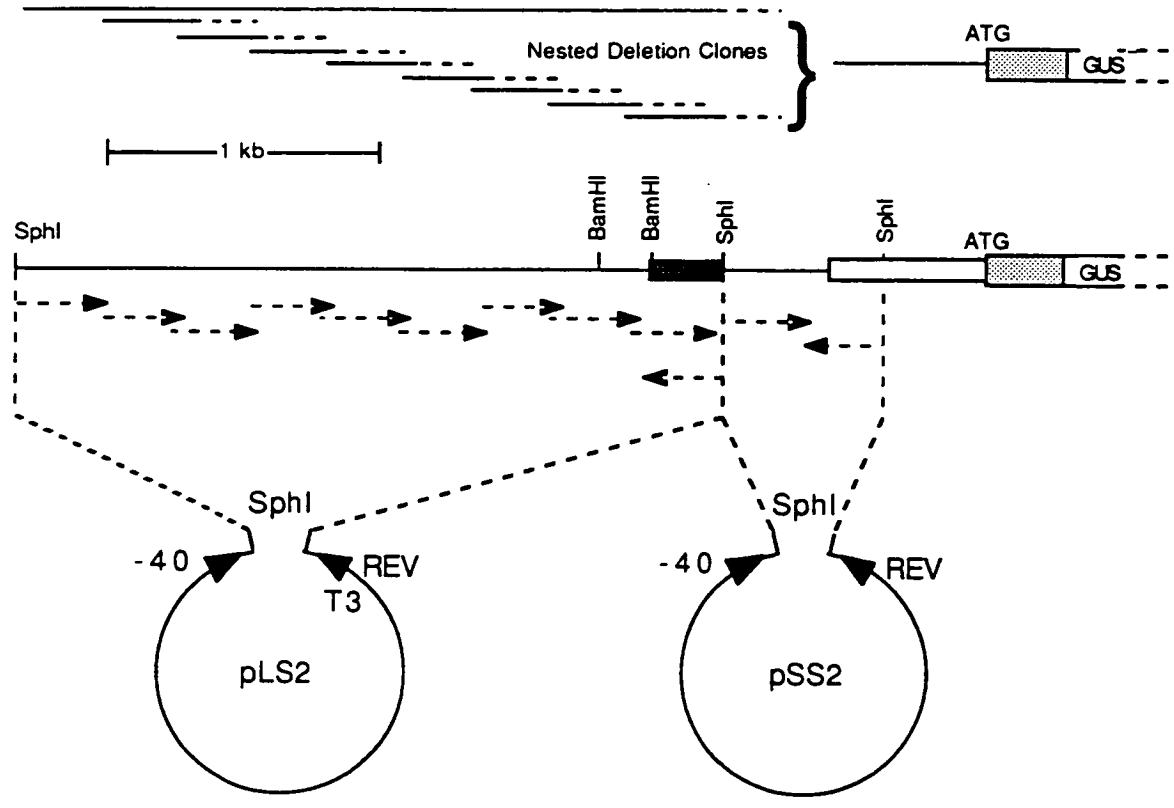
B A restriction map, cloning and sequencing strategy (similar to **A** above) is presented for pG1-GUS (2.7 kb of genomic sequence upstream of *ANT1* fused to the GUS reporter gene).

RNA (antisense) probes were generated from the pLS1 clone, which had been digested with BamHI, using the T3 promoter in the pBS+ cloning vector. These RNA probes were used for RNase protection analysis (see section 4.8.2)

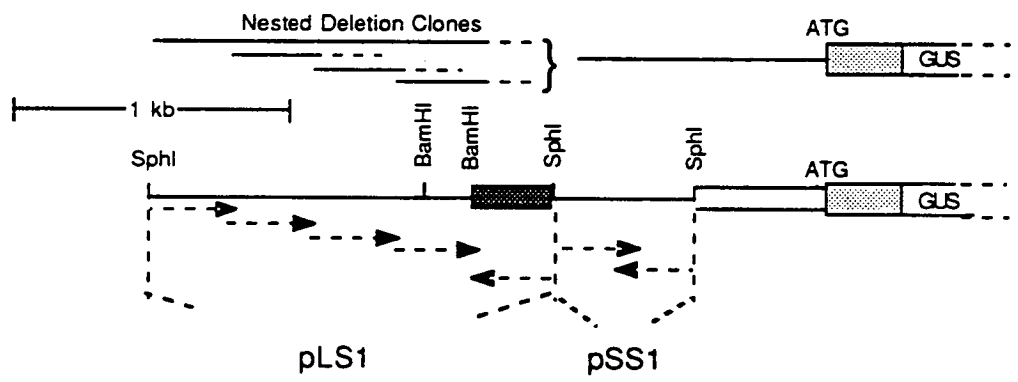
The symbols used in this figure are summarised below:

-  = coding sequence of *ANT*
-  = expected location of exon2 from the Southern blot analysis in Figure 5
-  = indicates the extent of the 5' region previously sequenced before this sequence analysis
-  = -40 or Rev sequences in the pBS+ cloning vector
-  = indicates the sequencing strategy

A



B



pBS+ -40 and reverse primer sites. To sequence the remaining internal region (intron A) nested deletions from the 5' end of the *ANT/GUS* fusion clones, pG1-GUS and pG2-GUS, were prepared using XhoIII (Figure 4.6). These deletion clones were constructed by Dr Joe Ross and allowed sequencing of intron A to exon 2. All the deletion clones were sequenced from the 5' end of the clone. Since the 5' ends of all the deletions were within intron A a series of overlapping sequences were generated (Figure 4.6). At the time (before intron A had been identified) these nested deletion clones were intended to be used as a deletion series of the promoter region for promoter analysis.

The sequence obtained for *ANT1* and *ANT2* is presented in Figure 4.7 and compared with pMANT1 and pMANT2 to show the location of exon 2 and the 5'/3' boundaries of intron B. Intron B from *ANT1* and *ANT2* has a similarity of 81% with 23 gaps which was determined using the computer programme GAP (section 2.5). The size of the intron is 1063 bp and 1021 bp for *ANT1* and *ANT2* respectively which is approximately ten times larger than intron C and intron D which separate the translated exons (Figure 4.3). The structure and role of intron B (and intron A) will be discussed later (section 4.9 and 6.4.2).

4.7 REINTERPRETATION OF TRANSCRIPT MAPPING BY S1 AND MUNG BEAN NUCLEASE ANALYSIS

Now that the location of intron B has been confirmed, within the new genomic sequence, the original transcript mapping data discussed in section 3.2.2 can be reinterpreted. The transcription initiation site predicted by Bathgate *et al.* (1989) by mung bean nuclease mapping and the site predicted by Baker (1985) by S1 nuclease mapping corresponds approximately to the 3' intron : 5' exon splice site (Figure 4.8). The original experiments correctly mapped the exon : intron boundary but this was incorrectly interpreted as the transcription initiation site.

Figure 4.7

Nucleotide sequence of the *ANT* genomic clones from the EcoRI restriction endonuclease site to the ATG translational start (boxed). Included in this sequence are the putative introns A, exons 2, introns B and the 5' ends of exons 3 of *ANT1* and *ANT2*. Above the *ANT1* sequence is nucleotide sequence corresponding to the pMANT1 cDNA clone. Below the *ANT2* sequence is nucleotide sequence corresponding to the pMANT2 cDNA clone.

Intron B splice sites and the putative branch sites are compared with the consensus sequences proposed by Brown, (1986).

The *ANT1* and *ANT2* sequences complementary to the specific oligonucleotides used for primer extension and RNA sequencing analysis are boxed. The primer extension products derived from these oligonucleotide primers are identified by a vertical line and a number. The number refers to the size, in nucleotides, of the extension product (see section 4.8.1).

The 5'-ends of exons 2, predicted by RNase protection analysis, are marked by the symbol \oplus for *ANT1* and *ANT2* (see section 4.8.2). The antisense RNA probes correspond to the genomic sequence between the BamHI restriction endonuclease site (1093 bp for *ANT1* and 2282 bp for *ANT2*) and the SphI restriction endonuclease sites (1285 bp for *ANT1* and 2560 bp for *ANT2*).

ANT2.....GAATTCGT 8
 9 AGAAGCCATTGTGGATACGGAACGCTGTTTGTGATGTCAGCTAGACGC 58
 59 TTGGCGACTTGATGATAGCCCAAGCGTAGGTCGGGTTTGGTGAAGAAGCTT 108
 109 GGCACCATGGAGCTCGTCAATCAGCTCGTCACTATGGGGATGGGGAAGGC 158
 159 ATCCTTGATGGTATGGCGTTCAAGGCGCGGTAGTCGACGCAGAACCCGC 208
 209 ACCGGCCATTAGGTTTCTTGACGAGGAGGACGAGCGAGGAGAACCCGAG 258
 259 GAACTCGGGGACGAGCCTTGCTCGAGCATGGCGATGCACTATCGCTCCA 308
 309 GCTCGTCTTGTGGGACTGGATAGCGGTAGGGGCCATCGCACAGGCT 358
 359 TCGAGCGTGGACGAGAGTGTCTGTGTCGTGGGAGCGTGGCGGTGGGA 408
 409 GCCCCCGTGGTTCAGAGAAGACGTCGGTGAAAGACGCGAGTAGCTCGTCT 458
 459 AGTAGGGATGGTGACCCGAGGTGGTGGCGAGGGTGGCGCCACATTCC 508
 509 CTTGGACGCCATGCCAGCAGATGGCTGGCCCTGCCGATAGAATGTTAGT 558
 559 TTCTGGGCGCGAAATCCTAGAGGACGACCCCAACGTGGCCAGCCAAGC 608
 609 AGTGCCCAACACCATGTCGAAACCTGCAAGTGGCATTACAACAGGTGAG 658
 659 CSTGCAAGGTGGCGACATGGATAGTGAATGGACCTGCCGGAGACGCCCA 708
 709 CGCAGGTGACGGTTCACCGTTCGCCATTGTTGCCGTAGCCGCGACCGA 758
 759 CTTTGGAGAGGACGCCCGGTGCGTCTTGGCCGTGGACTCGGATATGAAT 808
 809 CATGTGTCGACC CGGAGTCGAGTAGCGCTAGAAGAGGGGAGGCTCCCAAC 858
 859 ATGACGGCGACCTGCATCGTCCGCTGAAGCAGCCCTCGGAGGGTGCAG 908
 909 GGAAGAGCGGGAGTCTCCGCTCCGTTGTCGTACCGTCTGTCGATGT 958
 959 CCACTCCATGGACGTAGAAGAGTCGCTTGCAGGTGCGGTGTGCCCCAC 1008
 1009 GTATATTTCTCGTCGAGTGAAGCACAGGCCAACCAGTACGCTCTCTG 1058
 1059 CTCGGGCGCGATAGCGCTCCTACTGGTGGCCCTCCGTGGCCGCGGTGTA 1108
 1109 GCCGTCGGTCTCCTGGGCCACAGAG..AGGGCGAGACATCAACGATCA 1158
 1157 CCAAAAATCTAAGGCCATCTCAGTTGGAAAGTACTGTACTTATTG.AAC 1205
 ANT1..GAATTCGGCTGTGGGAGAACTAGTCGTAGAGAGAATTTAGATGTGAA 49
 1206 TTTAATCAACGATCACCAAAAATCTAAGACTATCGTAGGTGTCTGAGGA 1255
 50 GAGGAAGAT.AAAGAGGTGCATATGGTTT.....ATGATTTAGAAAG 90
 1256 CAGGGAGCTAAAATAGTTTTATATGGCATTCTCAAGAATGTTAAAGTAGA 1305
 91 TGACGAGTTCTTATTAACCTGATGACTCGGTGATTTGATGTTTATGTTG 140
 1306 TTAGGAGTCATTAGGGTATTGATTAGTCACTACA.TATGGTCCATTGTG 1354
 141 ACTTTAGACGGTTTTTACTAAATTAATTTTACAAAAGTGGCTAAAAAG 190
 1355 GTATGAGATTTTGGAGTTGGTAATGGAGTCGTGACAAGGCTGATTGAAG 1404
 191 ATGGACGTTTGATAATCTTCAGCAACTTCTGGTTGCGAGAAGTTAAAAAA 240
 1405 AAACAATTATGTCAGAGGTAACCTTCAGCCGGGTGTCGAA..CACACCC 1452
 241 AACTGAAACAAACCGAACAAAAAATGTTACATGCTTTTCTCACAAAAAC 290
 1453 GCCTAATGCCAAGAACAAAGGGGTTGTCAGGGTTAGCCCTAATGAGGTG 1503
 291 TACACATTGACTCGACATCTTAGTGTAGACTGTAGTACAATGCCTTCCCG 340
 1503 TAAGAACACACCGAGGATTTAGAGTGGTTCGGGCCACCAGAGTGAATAC 1552
 341 TTGACCTCAAGAAAATGCAATTCATGGAGCACTCCAATTCGATGTTTTCC 390
 1553 ACTACGTC..TATTATGAGTTGATTGCTAAGAGTTTGGCATGAATGAA 1600
 391 TACTTCGCAGGCACTCACTTTCCTTCCCACAGCTAACCTCTCCACAA 440
 1601 GATGTGTATGACCCTAAATGAGGATGCCCCITTTGTAACACATGTGCATC 1650
 441 T.TCCAATTTCCAAATGTTGCGTTTTTCTTTTTTACAACATCCATCCGACT 490
 1651 TCTTTTATAACCCAATGGTACTCTCTAGAGTGCAAACAAGATATAGAAA 1700
 491 CGGAAGAACTAAAATCTCAGCGCCCTTCCCTTGCCTTGTGAGATCAAG 539
 1701 AGTTAGAAAAAGTTACTAAAATTAATAAATATAGAAAATAATTTTTATAC 1750
 540 AATGGACAGTCCAAAATCAGAAGAATTTAGCAATAAACACAGTGTAAAT 589
 1751 ATATGACTCAAAAAATAATTAGAGAGTGAAATTTAGAATAATCTTGGGA 1800
 590 GAGCAGATCCAGTCCCTACCAGTGTGAAGATGTCCTCAAAAAAATCAGA 639
 1801 GATGCTCTAAAG....TTATAGGACTCTATTAGGCTTCTATTATATTCA 1846

640 AAGAGACGAAAAATGCTGATACATACAGCCTAGTAGTGTCTCACTGCTGA
 1847 TTTTTTCTGTTAATCTGATTTCTAAAACATAAAATT.....
 690 GCGCTGACCATGCATCCATCACTTCGTGAAGACCACAACCAACTTGCAC
 1884 GAAATAACTAATGGTTAAATCGSTTTTGATTAAATAGATTTTATTTTCG
 740 GTTTCGCACACCCCTACCCGGCAAGCTCATGTTCGGTGTGCCCTTTCAC
 1934 GTTTCAAATTTATTTGTCTGC.....TGGTGGCTGCTCGCTTCTCTC
 790 ACCTCGCCTTCCCCAACCTTCGGTAGCGCTGGACCTGGACCCGATCTG
 1977 CAAACTTCTGGCTAGCGCTGTGCCTAGACCTCGACGTGGACCCGATCTA
 840 A.CCAACCCACCCACATGGAGTGGACATCCACCGTCCGTTCCAGCCTA
 2027 ATAAAAACCCACATGGGGGGTGCACATCCACCGTCCGTTCCAGCC.C
 889 AACAAACCCCTAAAGTCCGGATCCGGCTGGCACACTATACGGCTGC
 2076 AACAAACCCCTAACGCCATGGATCCGCTGTGCCATACCGTACACGGCCGC
 289
 939 TGTATTCCCCACCAACACG.....TCCGATCCCTCCGCTAGCCGAA
 2126 CCCCATATACCCCGACGGCCACGATCATCCGATCAACCGCTTCCGAA
 262
 981 CCGCCGTTCCGTTTCGACAACTCCGACCCGATCAGCCTACCCGGCGAA
 2176 CCCACCGTTCGGGGTCTGCTACTTTGCACCGGATCCGCTTTCGGGGCGAA
 203 165
 1031 TCGAAACAACCCAGCATCAGCATGGAACCAACCCGATGGGCCACCCGCG
 2226 TCAAAATGCC.....TGGCCTGGGACCCCGCATATGGCCACCCGCGC
 BamHI 126 115
 1081 GGGGGAGACGGCGATCCACCCGGCCCGCCGATCCGATGGCCAGCAGCGC
 2270 CGGGAAGACGGCGATCCACCCGGTCCCGCCGATCCGACGGCCGGCCCGG
 105
 1131 CGCGATTATAGCGTTGTGGTACGGCCGGCGGGCCGAGCGGAGATAA
 2320 CGCGA.....GGAGCCACTAGCGTGGTGGCGCGCTCGCGGAGATAA
 101
 1 61 57 CGC
 1181 AAAGGATCCCCAGGAAGGATCGCCC.CCCTCTCACTGGCCTCGAGCG
 2362 AAAGGCTTACCCAGGAAGGATCGCCCTCTCTTTCTCAAAGTCCGGAG.
 1 G.
 4 GCCTCTGAAAGTTCGGAGCCCGCCGCTCCGCTCC...TCTCCGCT
 1230 GCCTCTGAAAGTTCGGAGCCCGCCGCTCCGCTCC...TCTCCGCT
 2411TCGAGCCCGCCGAGCGCCTCCCTTCCCGCCT
 2 TCGAGCCCGCCGAGCCGCTCCCTTCCCGCCT
 pMANT1
 ↓
 52 CCGTCCCCA....AGG ANT1
 1278 CCGTCCCCA....AGGGTGAATCTTGGTCCACCCAGGCTTCTCTCC
 2449 CCGTCCCCAAGCCAGGCTGATCTCGTCCCGCCAGGCTTCTTCCC
 40 CCGTCCCCAAGGCCAGG ANT2
 ↑
 pMANT2 GGTAAGT 5' CONSENSUS SPLICE SITE
 1323 GTCCTCTGTCGCCGATTTAAGGCCCATGCTCTCGTCTCTATTGTGGCGA
 2499 GTCCTCTGTCGCCGATTT.AGGCTCGTCTCTCGTCTCTATTGTGGCGA
 SphI
 1273 TTGGTCCGCGATCCGCTGCTTTGTGGTAGATAGATCTGCTGCACGTC
 2548 TCCGTCGCGATCCCTCTGT.....TATATAGATCTGCTCTCCCGCTC

1423 TCGGTTCGTACGTATA.....GGTTGGATTGTTCG
 2592 CGGTTCGTACGTAGATCGATTATTTCCGGTTTAGGCTGGATTGTT..
 1454 TGTGGTAGCCTCGCCGTTGGTCTCATTAGATCGGTGCTTCTCCGCTC
 2640 TGTGGTAGCCTCGCCCTTTGGAGTTCATTAGATCGGTGATTCTCGGTC
 1504 GGGTAAGGGCTATTGAGATTTCTTAGTGGATCGTCTGCTATACTTGAG
 2690 GGGTAAGGGCGTTGACTCTCTA.....GTTTCATCTATACTTGAG
 1554 ATCTACTCCGTTTCTCCGTTAGTGTGTGTGCATCGCAGTGGATTTATGT
 2733 ATCTACTCCGTTTCTCCATAG...GTGTGGCTGCAGTGGATTTAATGC
 1604 TCCGTTTGGCTG.....CTGTAGATCTTCTGTAACTTTGT
 2781 TCCGTTTCTCGTGGAGCCTTGGAAATATTAGATCTTTATGGAGCTTTT
 1643 AGATCGCGAGGTTA.TGGTTATTGGTTAGTCACTCGG.....TAA
 2831 AGATCGTGAATAATTGGTTGGTAATCAGGTAAGTGGGAAGAAAAATG
 1683 GTGAGTTTGGACCGCTGTTTCGTGATAGTGACCGTCCGCTTGTAGACTG
 2881 TCGATTTTGGACCGCTGTTTCGTGATAGTGCGTCCGCTTGTAGACTG
 1733 TTTGTGTTCTTGGCCGTTGGATGGCATCTGACGGCAGAGCCCGTCCAA
 2931 ..TCTGTTATGAGCGTGGATGGCATCTGACGGCAGGCGCCGCTCTCAT
 1783 AAATATTGGTCAAGTGTGAACTGTTTAGACTATATGCATATATCA
 2979 TAGTACTGGATAACT.....
 1833 TGTGAAGGCTTGTATTACCATGCTCCCTGCACAAGATCTGCTTCACTTG
 2995ACCATTGTTTCAAGTTTTCAGTCTTGAAGTACTGAGTAAT
 1883 TTCGTCCACCTTATACCTTGGCATCTATCATTTAATGGTTTGGTG
 3040 AGAGTGCACACCTATACTTTTGCATCTGTATGATGTTCTTCCGTTGTT
 1933 TAATTGAATTTTGGGATCATGATGTTTTCATATGTTATAAAATCTGTG
 3090 GTAATTAGATTTGAAATCATGGT.....AATCTTTG
 1983 AACGTTAAAATTAATGAAGTGTGAAACATGCTAAAATGACGGTATT..
 3122 AATGTTAAAATTTCTGAAGTGTGAGCATGCTACAATGGGCTGTCTGC
 2031 ..TAAAATAATGATTCGTTTTCATGATCTGTTCTTCTTATGATTG
 3172 TGTAATTTTATTGATTCATTTTATGATCTGTTCTATTTTATGATTG
 2079 CTATCAAACTAGAGTTATGGAACATGAAAATGTAATATCAATGTCTG
 3222 TTGTCAAACTAGAGTTATGGAACATGAAAATATAAATGTGATGTTG
 2129 TATAATTGCACT.....TTTATCTGTCATTGACTTAATGT
 3272 TATAGTGCATTCATGTTTATTTCTGCTAATAGTTTATCTGTTAGTGT
 2164 TTATACACAGGCTCTCTTTTCAATTAAGTGTGTTGAGCTTTGGCTTTA
 3322 TTGCACGGAGGCTCTCTGCTTCAGTATAGTGTCTAGCTTTGATTGTT
 2214 CTCTGTGAAACACAGAAAATAGTGTGTTCTGTAGCTTGTACTATAT
 3372 GT.....TACTCTATATTGAAACTGTA.....AAAT
 2264 AATATGCATCTTTTACACACAGTGTAGACGTGGAGATCTGTTATTCTT
 3398 ATACTCATCTTTTTCACAGGTCGGAGGCTTATTTTGTTCTCTTT
 pMANT1
 ↓
 65 ANT1 CTGAG : BRANCH SITE CONSENSUS CCAATAGTT
 ↓ A T
 2314 ATGCACTATCCTAAGTTGTGTCTTATATATGTTGCAGCCAATAGTT
 3448 ATGTAATGCTCAAGTTTGTGTGTTGACATTATGTTGCAGCCAACAGTT
 58 ANT2 CCAACAGTT
 ↑
 3' CONSENSUS SPLICE SITE : TTTGAGG pMANT2
 74 TTCAAGTGGCAATGCGGACCCAGGCTAACCAACCCACTGTCTTCATAAG
 2364 TTCAAGTGGCAATGCGGACCCAGGCTAACCAACCCACTGTCTTCATAAG
 3498 TTGAGTGGTAAATGCGGACCCAGGCTAACCAACCCACTGTCTTCATAAG
 67 TTGAGTGGTAAATGCGGACCCAGGCTAACCAACCCACTGTCTTCATAAG

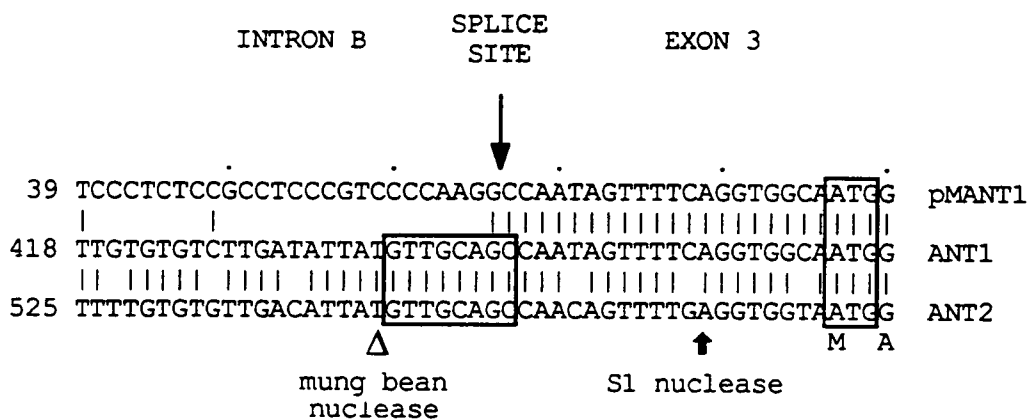


Figure 4.8

This figure has been adapted from figure 2.2. The S1 nuclease mapping (Baker, 1985) and mung bean nuclease mapping (Bathgate *et al.*, 1989) were originally interpreted as corresponding to transcript initiation sites. A reinterpretation of this data presented in section 4.7 indicates that the mapping actually indicates the location of the intron B : exon 3 boundary.

The boxed sequence at the splice site is similar to the consensus splice site sequence of TTTGCAG:G proposed by Brown, (1986).

4.8 IDENTIFICATION OF THE TRANSCRIPT INITIATION SITE FOR *ANT1* AND *ANT2*

4.8.1 Primer extension analysis to identify the transcript initiation sites for *ANT1* and *ANT2*

To identify the transcript initiation sites and the associated promoter sequences (now known to be intron A) for *ANT1* and *ANT2*, I initially used primer extension analysis. Two oligonucleotides complementary to 5' sequences (both 17 bp) were made by Oswell DNA Services, for the primer extension analysis, which were specific for the 5' sequences of pMANT1 and pMANT2 respectively (Figure 4.7). The oligonucleotides were designed so that the 3' ends were complementary to a highly degenerate sequence to ensure that the oligonucleotides would not misprime even if there was cross hybridisation.

The primer extension was carried out as described in section 2.4.8. For the *ANT2* specific primer extension, using RNA isolated from 3-day-old dark-grown seedlings, two extension products were produced that were shown to be 101 bp and 262 bp in length in comparison to the size markers generated by sequencing reactions (Figure 4.7, Figure 4.9). The 262 extension product is more clear in figure 5.5. These extension products correspond to 5' untranslated regions (5' UTR) in the *ANT2* transcripts with sizes of 159 and 320 bp.

Identical reactions were performed for the *ANT1* specific primer and up to 8 reproducible extension products were observed. Many of these bands may be spurious since some have much fainter extension products than the two major products. The 8 extension products have sizes of 57, 61, 105, 115, 126, 165, 203 and 289, the major ones being the 57 and 61 bp products (Figure 4.7 and Figure 4.9). These correspond to 5' UTR's in the *ANT1* transcripts with sizes of 124, 128, 172, 182, 193, 232, 270 and 356 bp.

Some of the faint extension products observed in the *ANT1* specific primer extension reactions may be the result of non-specific binding between the oligonucleotide primer and the *ANT1* transcripts. In an attempt to determine if the faint extension products were non-specific the annealing reactions were carried out at different temperatures up to 75°C. None of the extension products are reduced at higher annealing

Figure 4.9

Primer extension analysis to identify the transcript initiation sites of *ANT1* and *ANT2* using the gene specific oligonucleotides indicated in figure 4.7. The sizes of the gene specific primer extension products are indicated at the side and are derived from the sequencing reaction size markers in lanes 5-8. The size markers originate from a sequencing reaction using the *ANT2* genomic clone as a sequencing template and the *ANT2* specific oligonucleotide (also used for the primer extension) as a primer.

Lanes 1 and 12 Primer extension with cucumber 3-day-old cotyledon RNA.

Lanes 2-4 and 9-11 Primer extension with maize 3-day-old dark-grown (etiolated) seedling RNA.

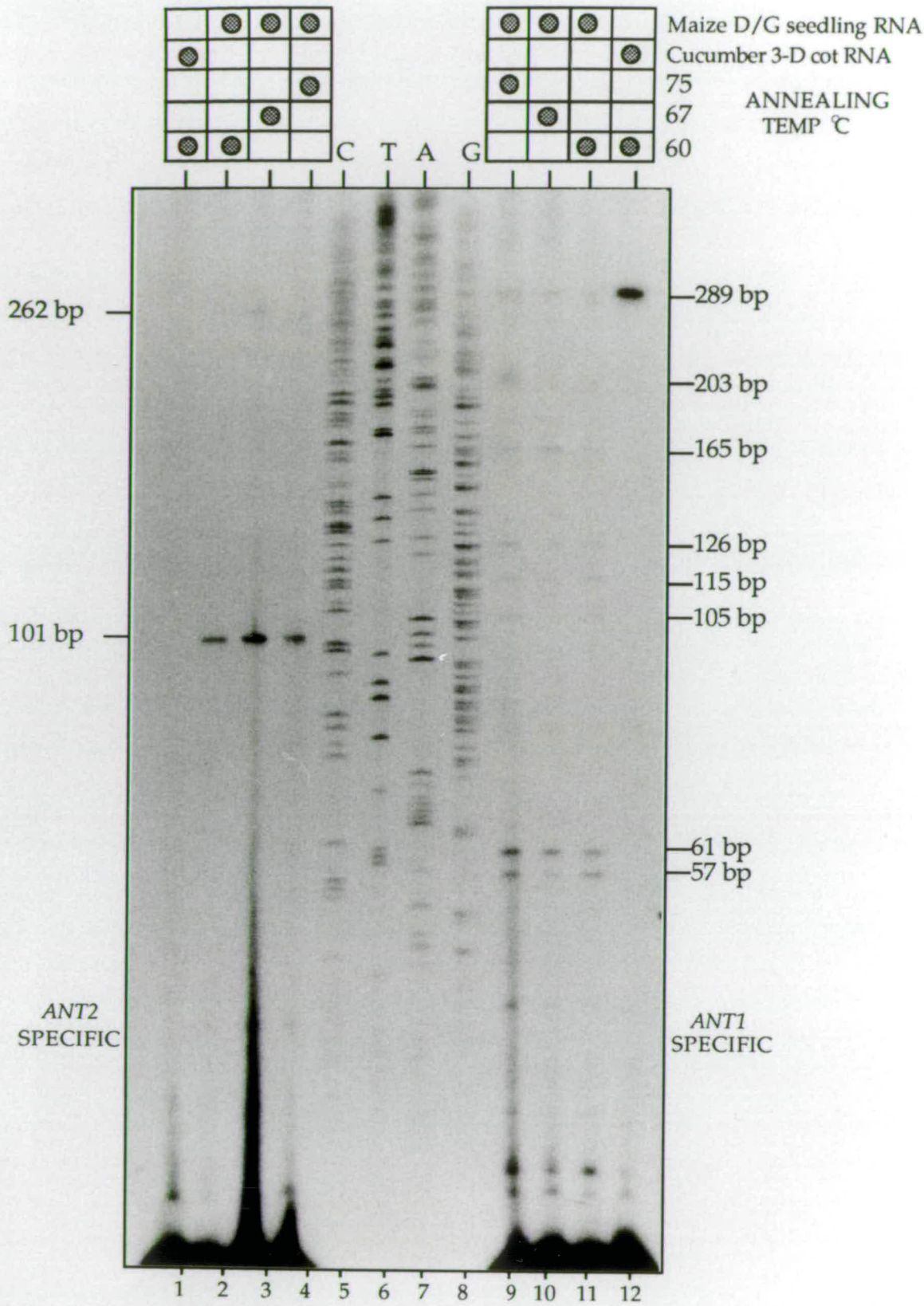
Annealing temperatures prior to the primer extension reaction were:

60°C for lanes 1, 2, 11 and 12.

67°C for lanes 3 and 10.

75°C for lanes 4 and 9.

C = cytosine, T = thymine, A = adenine, G = guanine.



temperatures which suggests that they are not due to non-specific binding of the pMANT1 oligonucleotide but are *ANT1* specific extension products. Another possible artifact which must be considered is that a stable 5' UTR secondary structure may cause premature termination and, thus, multiple extension products. If this were the case it is unclear why the *ANT1* 5' UTR secondary structure should cause more premature termination sites than the *ANT2* 5' UTR secondary structure. Presently much of the 5' UTR sequence is not known since exon 1 has not been cloned for either of the *ANT* genes (section 4.8.3). Consequently it is impossible to predict if 5' UTR secondary structure might cause premature termination. In an attempt to verify the primer extension products (exon 1 and intron A had not been identified at this time) I used an RNase protection assay (section 4.8.2).

4.8.2 RNase protection experiment to verify the transcript initiation sites

To confirm the primer extension analysis, an RNase protection assay was performed to map the various transcript initiation sites identified in section 4.8.1. The protection assay is described in section 2.4.9.

Antisense RNA probes used for the protection experiments were derived from the SphI subclones in pBS+, pLS1 and pLS2, as described in Figure 4.6. The RNA probes included some of the intron sequence (intron B) at the 5' end with the known exon sequence (exon 2) occurring in the middle of the probe (Figure 4.7 and 4.10). The 3' ends of the RNA probes are 5' of the major transcript initiation sites predicted from primer extension. During the protection assay RNase digests the 5' end of the RNA probe, which is complementary to the intron sequence, and any of the RNA probes 3' end which extends upstream of the transcript initiation site.

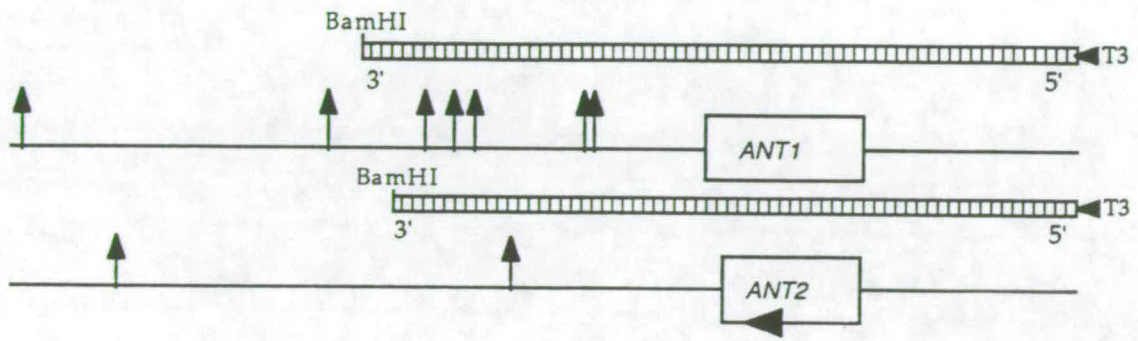
The antisense RNA probes were shorter than the largest observed primer extension products (Figure 4.10). If the faint, longer extension products were genuine all the 5' sequence from the RNA probes would be protected. This would have resulted in a fragment smaller than the full length probe, as the 3' end will always be digested. This is advantageous since complete protection of the 5' end will not be confused with any remaining undigested probe.

Figure 4.10

A graphical representation of the antisense RNA probes in relation to the *ANT1* and *ANT2* genomic sequences. The expanded sequence indicates the location of the 5' end of exons 2 as predicted by RNase protection analysis. The nucleotides highlighted in green are 3' splice site consensus sequences close to the region defined by RNase protection analysis.

RNA sequencing using the *ANT2* specific oligonucleotide primer (section 4.8.3) predicted that the 3' splice site is within a few base pairs of the of the primer. The only putative 3' splice site consensus sequences near the primer is a pyrimidine rich sequence highlighted in pink. These nucleotides are similar to the animal consensus of YYYYYYYYYNYAG: proposed by Senepathy, (1990).

- ▲ = indicates location of primer extension products
↑ in relation to exons 2 and the antisense RNA probes
- ◀T3 = T3 promoter used to make the RNA (antisense)
probe for the RNase protection analysis
- ◻C = cytosine complementary to the 3'-end of the
ANT2 specific oligonucleotide primer
- ◀ = ANT2 specific oligonucleotide primer
- ▣ = antisense RNA probe for RNase protection analysis
- ◻ = exon 2 sequence present in pMANT1/2
- = intron sequence or sequence which has not been
confirmed as exon 2



100 bp

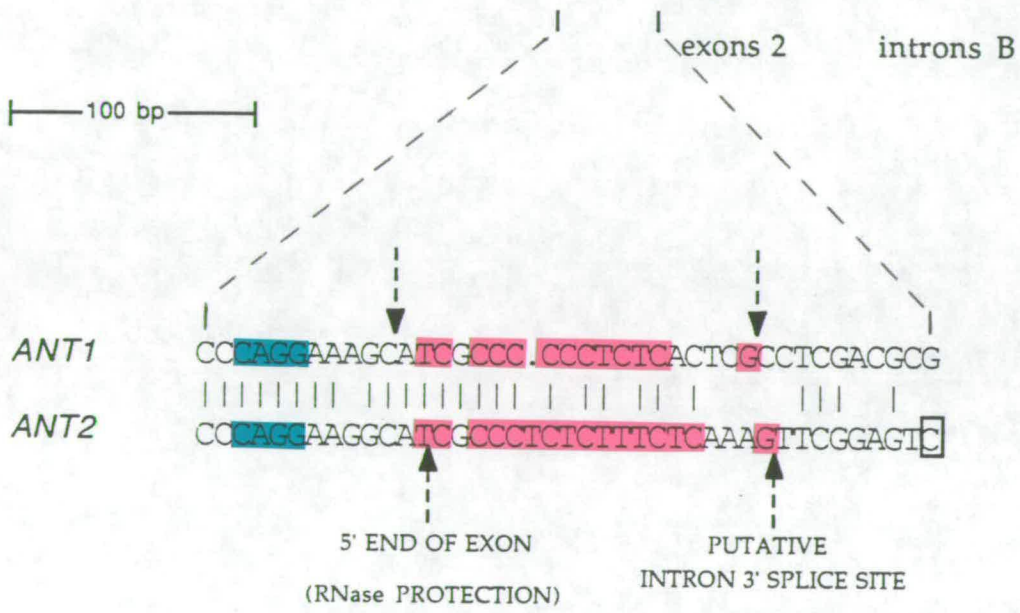


Figure 4.11

RNase protection analysis to identify the 5' end of exons 2 using antisense RNA probes synthesised from the plasmids pLS1 and pLS2 (Figure 4.6). The plasmids were digested with BamHI restriction endonuclease enzyme and the antisense RNA was synthesised using the T3 promoter. The antisense RNA probes were complementary to *ANT* genomic sequence expected to include exons 2 (Figure 4.10).

Sense RNA synthesised from the pMANT1 and pMANT2 cDNA clones was used as a positive control. The first 64 bp (pMANT1) and 57 bp (pMANT2) of the sense RNA are complementary to the *ANT1* and *ANT2* specific antisense RNA probes respectively. To synthesise the sense RNA the cDNA clones were linearised with HindIII (pMANT1) or KpnI (pMANT2) followed by sense RNA synthesis using the T7 promoter (Figure 4.4).

The sizes of the RNase protected fragments are indicated at the side and are derived from the DNA sequencing reaction size markers in lanes 5-8. The size markers originate from a standard sequencing reaction using the *ANT2* genomic clone as a sequencing template and the *ANT2* specific oligonucleotide (Figure 4.7) as a primer. The sizes in brackets represent the expected size of the positive control RNase protection and demonstrate that the RNA fragments run approximately 4.5% faster than DNA fragments of an equivalent size.

Lanes 1 and 12 Undigested antisense RNA probe.

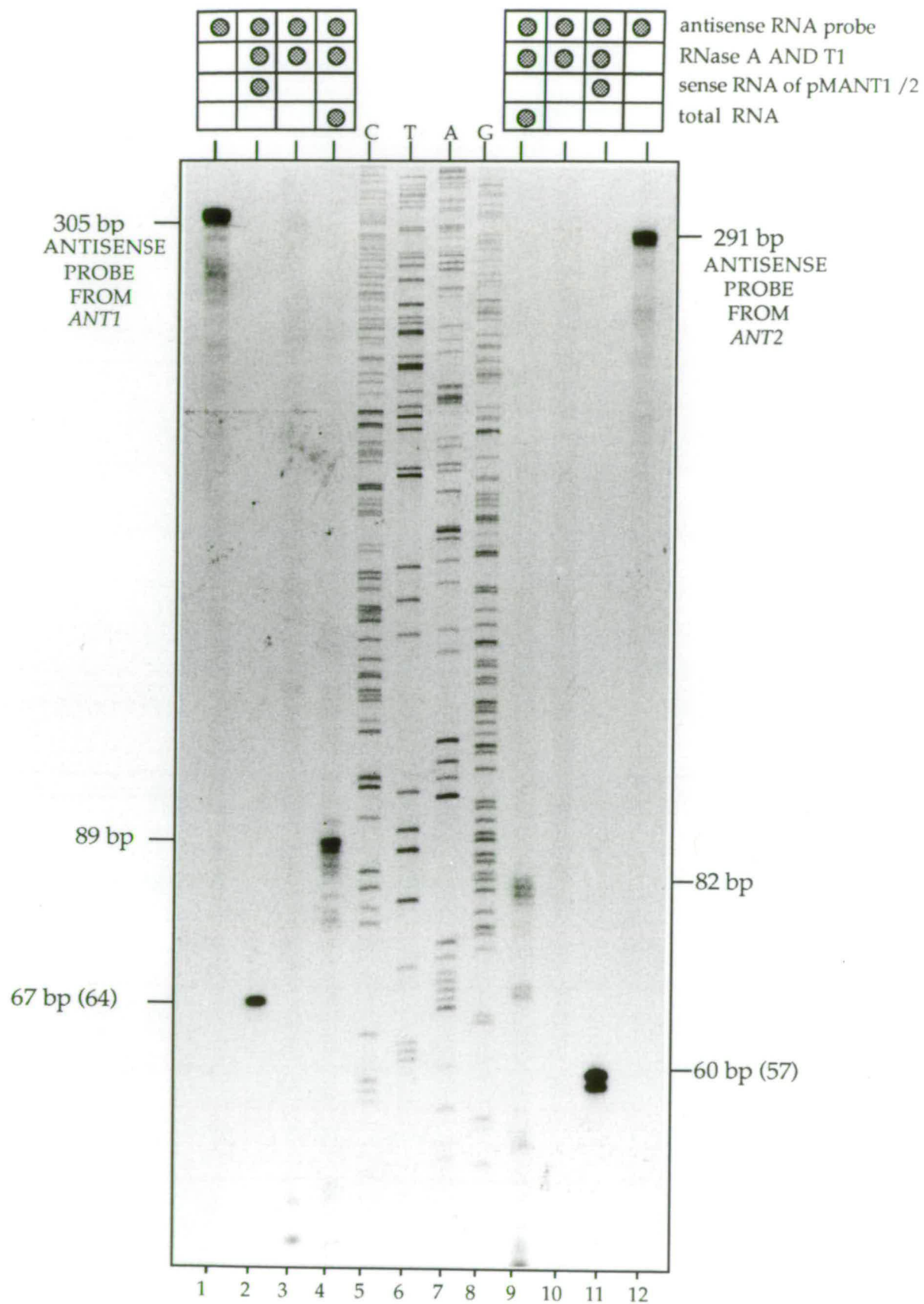
Lanes 2 and 11 Positive control, RNase protection of sense RNA/anti-sense RNA hybrids..

Lanes 3 and 10 Negative control, RNase digestion of the antisense probe.

Lanes 4 and 9 RNase protection of total RNA/antisense RNA hybrids.

Lanes 5-8 Sequencing reaction size markers; C,T,A,G

C = cytosine, T = thymine, A = adenine, G = guanine



As a positive control unlabelled synthetic RNA was made, from the pMANT1 and pMANT2 cDNA clones, which were complementary to the RNA probes. After a protection assay (substitution of the synthetic sense RNA for the total RNA) the protected fragments corresponded to the 64 and 57 bp 5' termini (exon 2 sequence) which are present in pMANT1 and pMANT2.

The DNA markers used were standard sequencing reactions using the *ANT2* specific oligonucleotide with the *ANT2* clone, pUG25, as a template. The DNA fragments migrate faster than the equivalent RNA fragments and therefore the sizes for the RNA fragments are an over-estimate. The difference can be calculated from the positive control in which the sizes of the protected fragments are known. For *ANT1* the protected RNA fragment from the positive control co-migrates with the 67 bp DNA marker but the RNA fragment is known to be 64 bp, approximately 4.5% less (Figure 4.11).

The largest protected fragments for *ANT1* and *ANT2* corresponded to DNA size markers of 89 bp and 82 bp but in both cases several smaller fragments were also present (Figure 4.11). Assuming the *ANT1* and *ANT2* protected RNA fragments were also 4.5% less than the DNA markers the final estimates for their sizes were 85 bp and 78 bp respectively. The smaller fragments observed could be due to the RNase nibbling into the complementary sequence, however, this is unlikely since the positive controls give distinct bands. The RNA used for the protection experiment was from 3-day-old dark-grown seedlings, a stage of development where both *ANT* genes are known to be transcribed (section 3.2.3). The smaller fragments were probably due to cross hybridisation of the RNA probes resulting in partial protection.

The sizes of the protected fragments did not correlate with any of the primer extension products (Figure 4.10). The sizes of the protected fragments predict exon sizes of 85 bp (*ANT1*) and 78 bp (*ANT2*) but the smallest exons predicted by primer extension analysis were 104 bp (*ANT1*) and 139 bp (*ANT2*). The evidence indicated that another intron (intron A) is located in the 5' UTR's with the 3' splice site defining the exon (exon 2) sizes predicted by the RNase protection mapping. This gene structure cannot be substantiated using the cloned cDNAs since neither extends far

enough to define the putative splice sites. A study of the genomic sequence revealed a putative intron 3' splice site consensus sequence in the region defined by RNase protection mapping (highlighted with green in Figure 4.10).

4.8.3 RNA sequencing of the *ANT2* transcript to confirm the existence of a second untranslated exon (exon 1)

To confirm the existence of a second untranslated exon (exon 1) in the *ANT* genes RNA sequencing was used to locate the 3' intron splice site and determine the sequence of the putative exon. The *ANT2* specific oligonucleotide was used for the RNA sequencing since the primer extension data indicates that the first major extension product is more intense than any using the *ANT1* specific primer (Figure 4.9). It is important that the signal is intense since it will be subdivided into the various sequencing products when the primer extension is carried out with ddNTPs. The RNA sequencing was carried out as described in section 2.4.7.2.

The sequence obtained was clear to the first extension product but subsequent sequence to the larger extension product was unreadable in the ddCTP and ddATP reactions (Figure 4.12). The sequence was compared with the 2390 bp of 5' sequence from *ANT2* but showed no similarity with any of the sequence. Consequently, if this putative untranslated exon is 5' of *ANT2*, the putative intron will be greater than 2.4 kb. In addition there is no similarity at the extreme 3' end of the RNA sequence with the *ANT2* sequence, which suggests the splice site is within a few base pairs of the oligonucleotide primer.

The putative splice site consensus discussed in section 4.8.2 and marked in green in Figure 4.10 and Figure 4.12 is not the splice site as there is no similarity between the genomic sequence 3' of the splice site and the RNA sequence. Analysis of the *ANT2* genomic sequence 5' of the oligonucleotide primer does not reveal a putative splice site consensus within a few base pairs of the oligonucleotide primer. The only possible consensus sequence close to the primer is an AG: 9 bp 5' of the primer (Figure 4.10 and Figure 4.12). Although this is not a good fit to the plant consensus of TTTGCAG:G (Brown, 1986) it does fit the animal consensus of YYYYYYYYYNYAG: as described by Senepathy (1990). A comparison

Figure 4.12

RNA sequencing of the *ANT2* specific transcript using the *ANT2* specific oligonucleotide (Figure 4.7) as a primer. The reaction was similar to primer extension analysis except each extension had one of either ddATP, ddCTP, ddGTP or ddTTP included in the extension mix. The sizes at the side of the lanes correspond to the primer extension products which occur without the addition of a ddNTP.

The column of nucleotides adjacent to the lanes is the predicted RNA sequence read from lanes C,T,A and G. The genomic sequence 5' of the *ANT2* specific oligonucleotide priming site is also presented for comparison. The boxed C is complementary to the 3' end of the oligonucleotide. The nucleotides highlighted with green and pink represent putative 3' splice site consensus sequences which are either side of the intron A:exon 2 boundary predicted by RNase protection analysis (Figure 4.10).

The lanes from left to right are:

Primer extension reaction (Control)

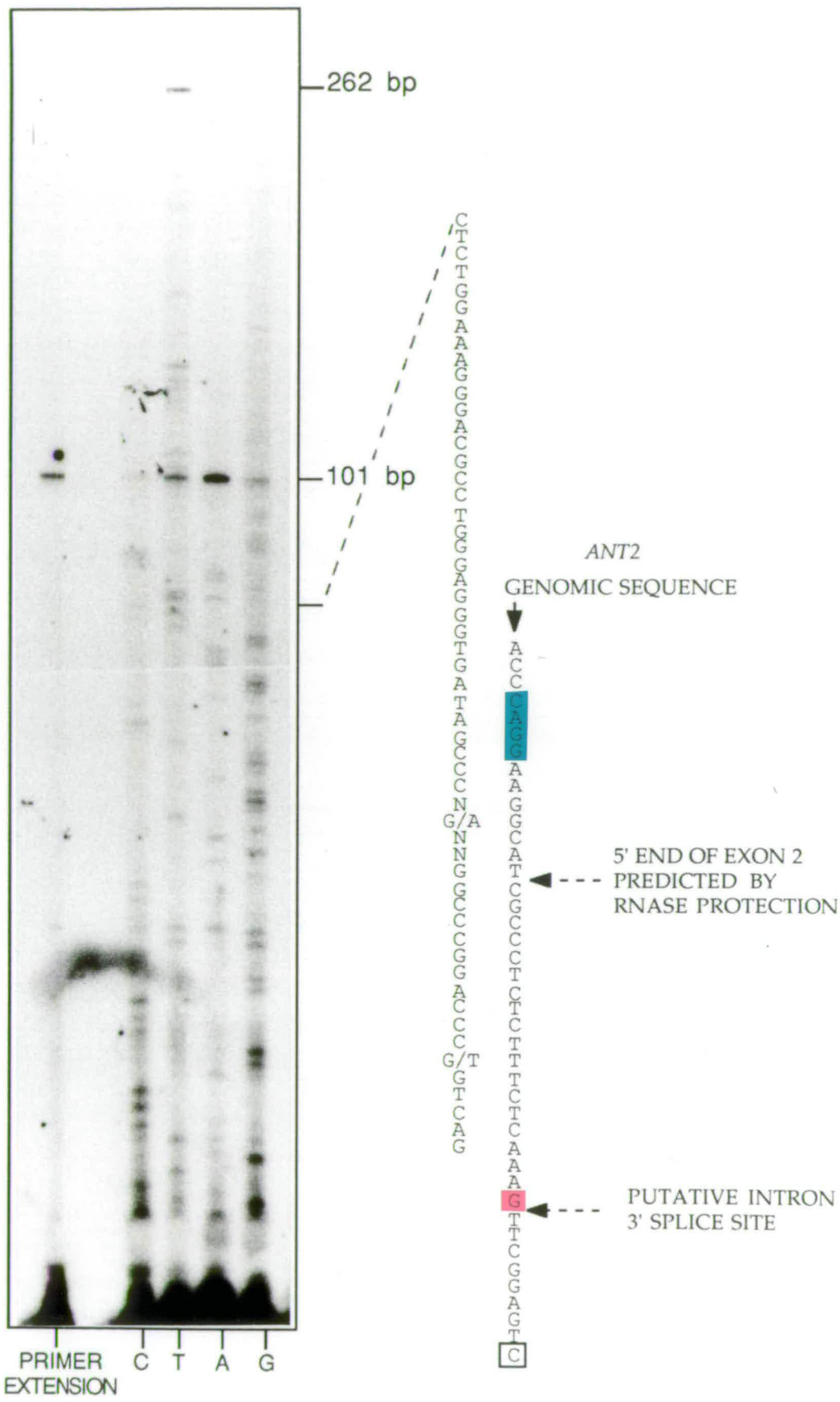
Blank

C Primer extension with ddGTP included in the extension mix

T Primer extension with ddATP included in the extension mix

A Primer extension with ddTTP included in the extension mix

G Primer extension with ddCTP included in the extension mix



of the *ANT1* sequence 14 bp down stream of the splice site predicted by RNase protection reveals a CG: sequence which is preceded by a pyrimidine-rich sequence (Figure 4.10). This non-consensus sequence is known to be an acceptable alternative to the ubiquitous AG: sequence (Jackson, 1991).

The RNA sequencing data predicts a location for the splice site within a few base pairs of the RNA sequencing primer. A putative splice site has been identified after analysing the genomic sequence in the region close to the primer. This putative splice site is 14 bp 3' of the splice site predicted by RNase protection experiments (section 4.8.2, Figure 4.10 and Figure 4.12). A possible explanation for this anomaly is that the RNA fragments migrate even slower than expected during the analysis of the RNase protected fragments by gel electrophoresis. However, 14 bp is a large error when the fragment is only about 80 bp in length.

Analysis of the genomic sequence does not reveal an obvious location for the splice site. To confirm the putative splice site further RNA sequencing should be performed with a pMANT2 specific oligonucleotide complementary to a sequence further downstream than the oligonucleotide primer used for RNA sequencing in this section. This experiment has not been performed as part of this thesis.

4.9 ANALYSIS OF THE INTRON SEQUENCES OF *ANT1* AND *ANT2*

A major difference between the monocotyledonous plants (monocots) and dicotyledonous plants (dicots) introns is the nucleotide composition. Goodall and Filipowicz (1991) have observed that monocots have an average AU nucleotide composition of 59% compared to 74% in dicots. AU-rich sequences present in the introns of dicot pre-mRNAs are required for splicing (Goodall and Filipowicz, 1989). It is hypothesised that the function of these AU-rich sequences during the splicing of dicot plant pre-mRNAs may be to minimise secondary structure within the intron (GC-rich sequences generally have a stable secondary structure in mRNA). To support this Goodall and Filipowicz (1991) have demonstrated that transiently expressed genes with either a short synthetic intron, with a stem loop, or a vertebrate intron (AU nucleotide composition of 45%) are spliced in maize (monocot) protoplasts but not in tobacco (dicot)

protoplasts. Additionally, Keith and Chua (1986) have shown that a maize and wheat intron (both about 62% AU nucleotide composition) are spliced inefficiently in transgenic tobacco plants. Another difference between the monocot and dicot introns is that the 20 bp 5' of the intron 3' splice sites are often pyrimidine-rich in monocots whereas in dicots they are more often purine-rich (Hanley and Schuler, 1988). In summary monocots are able to splice introns not spliced in a dicot system including vertebrate introns and, hence, monocots may differ from dicots in their splicing mechanism. Interestingly many of the structural features associated with the monocot introns are also characteristic of vertebrate introns, such as low AU nucleotide composition and pyrimidine-rich 3' splice sites (Reid and Steitz, 1985).

There are at least 4 introns in the maize ANT genes. Intron C and intron D had previously been identified by Baker (1985) and separate coding exons. I have identified a further 2 introns that separate the untranslated exons of both ANT genes which have been termed intron A and intron B (Figure 4.3). Intron A remains a putative intron sequence since the 5' and 3' splice sites have not been identified. The nucleotide composition of these introns and their splice site sequences are summarised in table 4.1.

Two introns in the 5' UTR of a gene is rare in all eukaryotic genes and no specific function is known for this gene structure (Gil *et al.*, 1986). Additionally the putative intron A and intron B are large introns in comparison to the average plant intron size of 249 bp (Hawkins, 1988). Intron A represents one of the largest known plant introns with a length of at least 2.4 kb in *ANT2* (Hawkins, 1988). A comparison of intron A from *ANT1* and *ANT2* reveals significant similarity from the predicted 3' splice site to a region approximately 400 bp 5' of the 3' splice site after which there is no significant similarity (Figure 4.7). Intron A has a particularly GC-rich (49% and 52% for *ANT1* and *ANT2* respectively) nucleotide composition although as discussed above this is not untypical of monocot intron sequences. Nevertheless a GC-rich sequence is characteristic of coding sequences and a number of open reading frames in both orientations have been observed in the intron A sequences. To determine if any known genes were encoded in either intron A their sequences were compared with sequences in the EMBL data base. No significant similarities were discovered although this does not preclude

TABLE 1	5' SPLICE SITE		3' SPLICE SITE			AU CONTENT	INTRON SIZE bp
		(a) snRNA n/10		INTRON TYPE	(b) Py:Pu -20to-2		
CONSENSUS (c)	G:GTAAGT		TTTGCAG:G				
<i>ANT1</i> intron A	N.D.		(<u>TC</u> ACTCG:C)	(Y)	(15:3)	51	>1.2kb
<i>ANT2</i>	N.D.		(CTCAAAG:) <u>I</u>	(Y)	(15:3)	48	>2.4kb
<i>ANT1</i> intron B	G:G <u>T</u> GAGT	7	<u>G</u> TTGCAG:C	M	11:7	59	1063
<i>ANT2</i>	G:G <u>T</u> GAGT	7	<u>G</u> TTGCAG:C	M	10:8	60	1021
<i>ANT1</i> intron C	G:GTA <u>G</u> CA	5	<u>G</u> TCCAG:G	Y	14:4	64	94
<i>ANT2</i>	G:GTA <u>G</u> CA	5	TGCCTAG:G	R	10:8	68	174
<i>ANT1</i> intron D	G:G <u>T</u> TTGT	6	<u>C</u> TTGCAG:G	Y	15:3	66	81
<i>ANT2</i>	G:G <u>T</u> IIGC	6	TTTGCAG:G	Y	14:4	63	80

(a) Number of bases in 5' splice site that match snRNA sequence (TCCATTCATA) Hanley and Schuler (1988).

(b) Three types of 3' splice site based on the bases between -20 and -2 as defined by Hanley and Schuler (1988), M=mixed, Y=Pyrimidine rich and R=Purine rich.

(c) Brown (1986).

Table 4.1

A comparative table of the ANT introns A, B, C and D. The 5' and 3' splice sites are compared to the consensus sequences. Nucleotides which are not identical to the consensus sequence are underlined. The introns have also been grouped into classes according to their purine and pyrimidine content as described by Hanley and Schuler (1988). The AU content is the percentage of adenine and uridine in the intron RNA which is generally lower in monocots than dicots (dicot average is 74%). This is discussed section 4.9.

the possibility of an unknown gene in intron A. The AU nucleotide composition of the intron B is 59% and 60% for *ANT1* and *ANT2* respectively. This is similar to the average for monocot introns of 59% (Goodall and Filipowicz, 1989). Introns C and D have a higher AU composition but this is still lower than the average composition of 74% for dicot introns (Table 4.1).

The maize *ANT* intron splice sites are compared to the consensus sequences identified by Brown (1986). The *ANT* splice sites are similar but not identical with the consensus although the majority of the introns used to determine the consensus were of dicot origin. Hanley and Schuler (1988) have shown that the consensus splice sites may be different between the dicot and monocot introns. They suggest that the majority of monocot introns have a pyrimidine-rich sequence 5' of the 3' splice site while the equivalent nucleotides in the dicot introns are purine-rich. A study of the *ANT* 3' splice sites reveals, in agreement with Hanley and Schuler (1988), that the majority of these sequences do have a pyrimidine or mixed (pyrimidine/purine mixture) type of splice site. Only the *ANT2* intron C has a purine-rich sequence more typical of dicot plants. In general the AU nucleotide composition of the maize *ANT* introns and their intron splice sites are typical of other monocot introns cited in the literature (Goodall and Filipowicz, 1991, Hanley and Schuler, 1988). The possible functional roles of introns A and B in the 5' UTR are discussed in section 6.3.2 while future aspects of this work to identify the 5' and 3' intron boundaries of the putative intron A are discussed in section 6.4.

4.10 MULTIPLE TRANSCRIPT INITIATION SITES IN THE *ANT* GENES MAY INDICATE A 'HOUSEKEEPING' ROLE

There is a significant difference in the number and position of the transcript initiation sites between *ANT1* and *ANT2* (Figure 4.7). *ANT1* has an unusual gene structure since it has multiple transcript initiation sites. This is characteristic of so called 'housekeeping genes' such as human glucose-6-phosphate dehydrogenase (Martini *et al.*, 1986) and the mouse DHFR gene (Crouse *et al.*, 1985). These genes typically have 3 features:

- i) No TATA or CAAT boxes (which is thought to explain the multiple initiation sites).
- ii) A high G/C content in the promoter region.
- iii) G/C box motifs 5' of the promoter.

A general definition of a 'housekeeping' gene is that it is expressed in all tissues but not necessarily at the same level. The role of the ANT protein could be regarded as 'housekeeping' since many of the metabolic functions in a cell require energy from the mitochondria at all stages of development. ANT is vital to transfer the energy, in the form of ATP, from the mitochondrial matrix to the rest of the cell. A hypothesis to account for the difference in the primer extension products between *ANT1* and *ANT2* is that one gene is highly regulated during the different stages of plant development (*ANT2*) but the second gene has a 'housekeeping' function (*ANT1*). As discussed in future work (section 6.4) it is vital to clone and analyse the promoter sequences of ANT to determine if either gene has a promoter structure typical of a housekeeping gene.

CHAPTER 5

EXPRESSION AND REGULATION OF ANT AND ATP SYNTHASE DURING *ZEA MAYS L.* DEVELOPMENT.

5.1 RATIONALE

As discussed in Chapter 1, a plant cell's demand for energy varies considerably depending on the stage of development, differentiation or the prevailing environmental conditions. I have investigated the expression of genes encoding the ATP9, α and β subunits of the ATP synthase and ANT which are key components of the enzyme complexes involved in mitochondrial ATP synthesis and transport to the cytosol. Previous biochemical analysis has indicated that both of these enzyme complexes may exert significant control over the rate of oxidative phosphorylation (section 1.4.1.1). If the amount of oxidative phosphorylation is regulated during plant development it may be achieved by regulating either ATP synthase activity, adenine nucleotide transport by ANT or both. I used RNA molecular probes for genes of both proteins to measure transcript steady state levels in various tissues from maize to determine if there is evidence for transcriptional or post transcriptional regulation. In addition ATP9 and α subunit are both encoded in the mitochondrial genome whereas the β subunit is encoded in the nuclear genome. Consequently I have been able to determine if the nuclear and mitochondrial genes encoding subunits of the ATP synthase complex are coordinately regulated.

5.2 MAIZE TISSUE USED FOR RNA EXTRACTION

RNA was extracted from a variety of maize tissues from different developmental stages throughout the life cycle.

Examples of vegetative tissue are mature leaves, stem and adventitious roots which were all harvested from a mature maize plant. Two different root tissues were distinguished and harvested from 3-day-old dark-grown seedlings. The root tip, which contains the meristematic region was defined as the first 5 mm of the root. The elongation zone, which contains differentiated root cells, was defined as the region 10 mm from the root tip

to the seed. The etiolated leaves and coleoptile sheath from the 3 day-old dark-grown seedlings were also harvested.

Several different tissues were also collected from the female reproductive organs (the cobs) which developed on the mature maize plants. Two different husk leaves were harvested from the cob, the first husk leaf and the fourth husk leaf. Both husk leaves are photosynthetically competent but the fourth husk leaf is less photosynthetically active since the three exterior husk leaves bind it very tightly and exclude the light (Yakir *et al.*, 1991). The other three tissues collected from the cob were kernels prior to pollination, the centre of the cob and pre-emergence silks.

5.3 QUANTIFICATION OF ANT TRANSCRIPT LEVELS BY NORTHERN BLOT ANALYSIS

The aim was to quantify the steady state transcript levels, using northern blot analysis. Three ways of standardising expression levels between different maize tissues were considered. These are:

- i) Probing each northern blot with a constitutively expressed gene to act as a standard between tissues.
- ii) Northern blotting the same quantity of RNA from each tissue calculated by measuring the concentration with a spectrophotometer.
- iii) Normalising the results to the amount of cytosolic rRNA as a standard between tissues.

The third method was eventually used although all three methods have disadvantages.

The first method is commonly used to compare transcript levels between different tissues. Actin genes are commonly assumed to be expressed constitutively (Barker *et al.*, 1988; Kagawa and Ohta, 1990). If these genes are used as a standard to compare expression levels between different tissues it must be assumed that they are expressed at the same level in all cells. This is probably an invalid assumption since apparently constitutively expressed genes are now known to be controlled by several regulatory elements (Benfey and Chua, 1990). In the various cell types

different elements and various numbers of elements combine to control transcription. It is therefore not reasonable to assume that the level of gene expression will be the same in all cell types.

The second and third methods are based on a comparison of specific transcript levels standardised to the amount of cytosolic rRNA in the sample. The second method is based on loading an identical amount of nucleic acid, which is measured using a spectrophotometer, in each lane of an agarose gel. Therefore it can be assumed that the specific transcript levels are standardised to the amount of rRNA which contributes to about 80-85% of the total. This method is not favoured since it is not possible to distinguish between the cytosolic and organelle rRNA. The variation of the chloroplast rRNA is large between the different tissues. In the photosynthetic tissue the amount of chloroplast rRNA can constitute a significant proportion of the total.

The third method uses the amount of cytosolic rRNA as an internal standard. There are two methods to quantify the amount of cytosolic rRNA. The first is to probe the northern blot with an 18S ribosomal specific probe. This method was not used since the large excess of rRNA on the blots may hinder the access of the probe and hence give a non-quantitative result. The second method involves photographing the rRNA bands which are stained with ethidium bromide and visualised using a transilluminator. The amount of cytosolic rRNA is measured by scanning the negative with a densitometer as described in Appendix I.

Ideally I would like to measure the transcript levels on a per cell basis. I have attempted to do this by using cytosolic rRNA as a standard between cells although the assumption that individual cytosols contain the same number of ribosomes is not necessarily valid. Also, the tissues chosen for northern blot analysis contain many different cell types. The steady state levels observed are only an average of all the cells in one tissue.

5.4 ANALYSIS OF ANT GENE EXPRESSION

As described in chapter 4 there are two ANT genes, *ANT1* and *ANT2*, which have multiple and distinct transcription initiation sites. Three approaches were used to study the expression of these ANT transcripts.

Firstly, northern blot analysis using a probe which is specific for both *ANT* transcripts to determine if expression is tissue specific. Secondly, northern blot analysis using probes which are specific for either *ANT1* or *ANT2* to determine if there is differential expression in any tissue. Lastly, primer extension analysis was used for *ANT2* to determine if the different transcription initiation sites are utilised at different stages of development.

5.4.1 A summary of the various probes used for the northern blot analysis of *ANT* transcript levels

5.4.1.1 A universal probe for the maize *ANT* genes derived from pMANT2

The universal RNA probe, for northern blot analysis, was derived from the pMANT2 plasmid (Figure 4.4). The plasmid was linearised with the PstI restriction enzyme and the T3 promoter, at the 3' end of the cDNA clone, was used to make an antisense RNA probe (section 2.4.4.2). The probe had 91% sequence similarity to the pMANT1 cDNA and hybridised with a similar specificity to sense RNA made *in vitro* from pMANT1 and pMANT2 (Figure 5.1 and following paragraph). Northern blot analysis with this probe gives a measure of the combined steady state level of *ANT1* and *ANT2* in the various tissues.

The sense RNA for testing probe specificity was synthesised from pMANT1 and pMANT2 (section 2.4.4.2) which had been digested with HindIII and KpnI respectively (Figure 4.4). The synthetic RNA was treated with DNase to remove the DNA template, thoroughly extracted with phenol:chloroform and precipitated with ethanol several times. The synthetic RNA was then resuspended in sterile Milli-Q® Plus water and its concentration calculated in a UV spectrophotometer (section 2.4.1). Stock solutions of pMANT1 and pMANT2 sense RNA were prepared with equal molarity. Serial dilutions were made from the two stock solutions. Aliquots of these dilutions were then slot blotted onto Hybond-N membrane such that equal concentrations of sense RNA from both pMANT1 and pMANT2 were adjacent and decreasing in equal dilutions down the filter. These control filters were utilised to test the specificity of the universal probe (above) and probes complementary to the 3' untranslated regions of the *ANT1* and *ANT2* cDNAs (section 5.4.1.3).

Figure 5.1

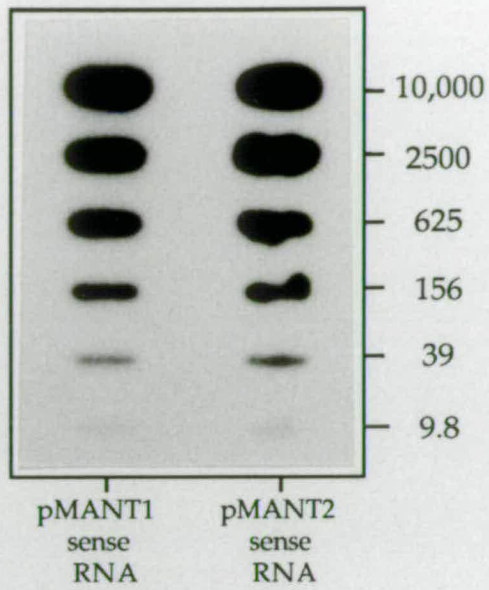
The universal *ANT* RNA probe, *ANT1* specific and *ANT2* specific RNA probes were hybridised to synthetic sense RNA from either the pMANT1 or pMANT2 cDNA clone to test the specificity of the probes. The numbers to the right of the hybridisation signals represent the relative quantities of sense RNA passed through a slot blot apparatus on to the Hybond-N membrane.

The synthetic sense RNA was synthesised using the T7 promoter from pMANT1 and pMANT2 which had been digested with HindIII and KpnI respectively.

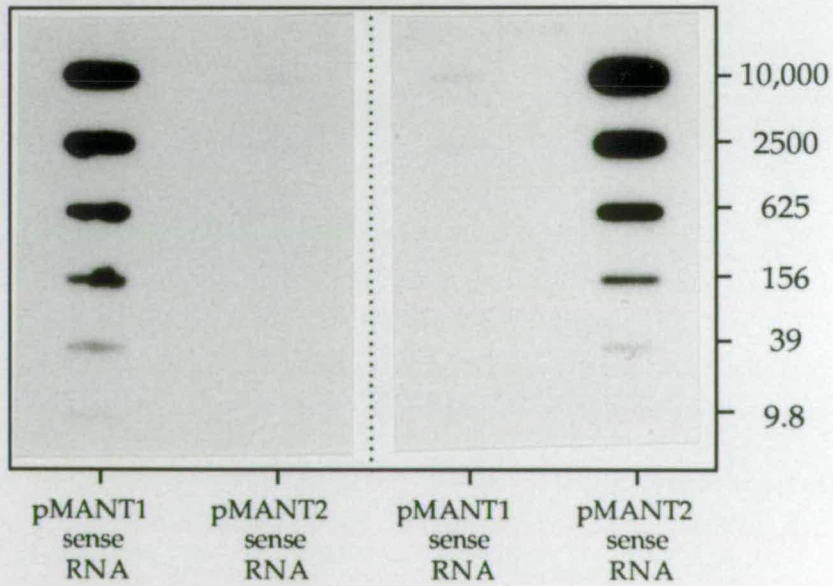
The universal *ANT* RNA probe was synthesised using the T3 promoter from pMANT2 which had been digested with the PstI endonuclease restriction enzyme (Figure 4.1 and 4.4).

The *ANT1* and *ANT2* specific probes were complementary to the 3' end of the cDNA clones. They were synthesised using the SP6 and T3 promoters from pMANT1 and pMANT2 (linearised with Sau3A) respectively (Figure 4.1 and 4.4).

The intensities of the hybridisation signals detected on the exposed film by densitometry are presented in Appendix III.



ANT universal probe



ANT1 3' RNA probe

ANT2 3' RNA probe

5.4.1.2 Gene specific probes derived from the 5' untranslated region of the *ANT* cDNAs

DNA or RNA probes to the 5' untranslated region of *ANT1* or *ANT2* cDNAs were synthesised using the 5' subclones previously described in Figure 4.4. RNA probes from pG1-64 and pG2-51 show an approximately 60 fold differential specificity for the respective genes (data not shown)(section 4.5). DNA probes, derived from the same plasmids, were made by isolating the insert DNA and random priming (section 2.4.2.1). These pG1-64 and pG2-51 DNA probes have a differential specificity of approximately 120 and 16 fold respectively determined by hybridisation to a Southern blot of known quantities of the *ANT1* or *ANT2* genomic clone DNA (data not shown).

The DNA and RNA probes were hybridised to northern blots of maize root and 3 day-old etiolated seedling RNA. The expected 1.6 kb *ANT* transcript was observed with other transcripts (Figure 5.2). The low specific activity of these probes and the excessive non-specific background made them unsuitable for the analysis of differential expression.

An intriguing problem which has arisen from using these probes is the nature of the smaller bands. Whether they are due to specific or non-specific hybridisation has not been established. If specific hybridisation is occurring between these probes and other transcripts the 5' untranslated region of the *ANT* genes may have a functional role which is shared by other unknown transcripts. The current EMBL data base was searched with the *ANT* 5' untranslated sequences to identify possible candidates for the unknown transcripts. No genes with significant similarity to the untranslated sequences were identified in the EMBL data base but significant similarity with a human *ANT* and a maize *Shrunken-1* sequence have been identified by inspecting published sequence data (section 6.3.1.1 and 6.3.1.4).

5.4.1.3 Specific probes derived from the 3' untranslated region of *ANT* cDNAs

Digestion of pMANT1 and pMANT2 with *Sau3A* restriction enzyme provided the templates to make 3' end RNA probes for both maize *ANT*

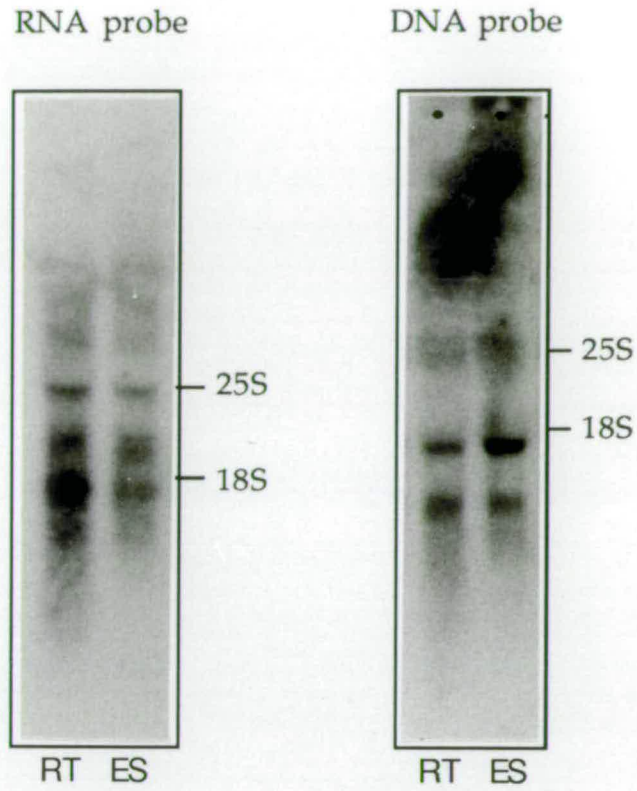


Figure 5.2

Northern blot analysis of RNA isolated from either root tips (RT) or etiolated seedlings (ES). 10 mg of total nucleic acid was loaded in each well. The probes were DNA and RNA probes derived from pG2-51 which contains the 5' terminus (51 bp of the 5'-UTR) of the *ANT2* cDNA clone pMANT2. The markers are ribosomal RNA's from maize.

genes (see Figure 4.1, Figure 4.4 and section 4.5.5). To test their specificity each probe was hybridised to sense RNA of pMANT1 and pMANT2 which was immobilised on Hybond-H membrane. The pMANT1 (1403-1579 bp) and pMANT2 (1390-1511) 3' untranslated probes were found to have differential specificities of approximately 650 and 370 fold respectively by hybridising to known quantities of sense RNA specific for *ANT1* and *ANT2* which was immobilised on Hybond-N membrane (section 5.4.1.1 and Appendix 1)(Figure 5.1).

The 3' probes are larger than the 5' probes, have a greater specific activity and less non-specific binding. In addition, the 3' probes have a greater transcript specificity than the 5' probes. For these reasons 3' RNA probes were used to analyse the differential expression of the maize *ANT* genes. A potential disadvantage is that not all *ANT* transcripts may be as long as the pMANT1/2 cDNA clones. For example, the cDNA clone pANT1 has a much shorter 3' untranslated region than pMANT1 (see Figure 4.1 and section 4.5.5).

5.4.2 Tissue specific expression of *ANT*

The protocol used for the northern blot analysis is described in section 2.4.5.2. After hybridisation with the non-specific probe (section 5.4.1.1) *ANT* transcript steady state levels were quantified for each tissue by densitometry as described in Appendix I. Each signal was standardised using the sense RNA control filters to estimate the film response curve (section 5.4.1.1 and Appendix I). This is important for an accurate calculation of the transcript ratios since the film rapidly becomes saturated and, hence, responds non linearly to radiation. A summary of the data obtained by northern blot analysis is presented in figure 5.3.

It is apparent that *ANT* transcript steady state levels vary between the different maize tissues over a 10 fold range. The strongest signal being root tip tissue, the weakest signal being leaf tissue. Only one transcript size, of approximately 1.6 kb, is observed despite the variation in transcript initiation (section 4.9.1 and Figure 4.9). The lower band of hybridisation observed in the leaf and husk leaf tissues is non-specific binding to chloroplast RNA.

Figure 5.3

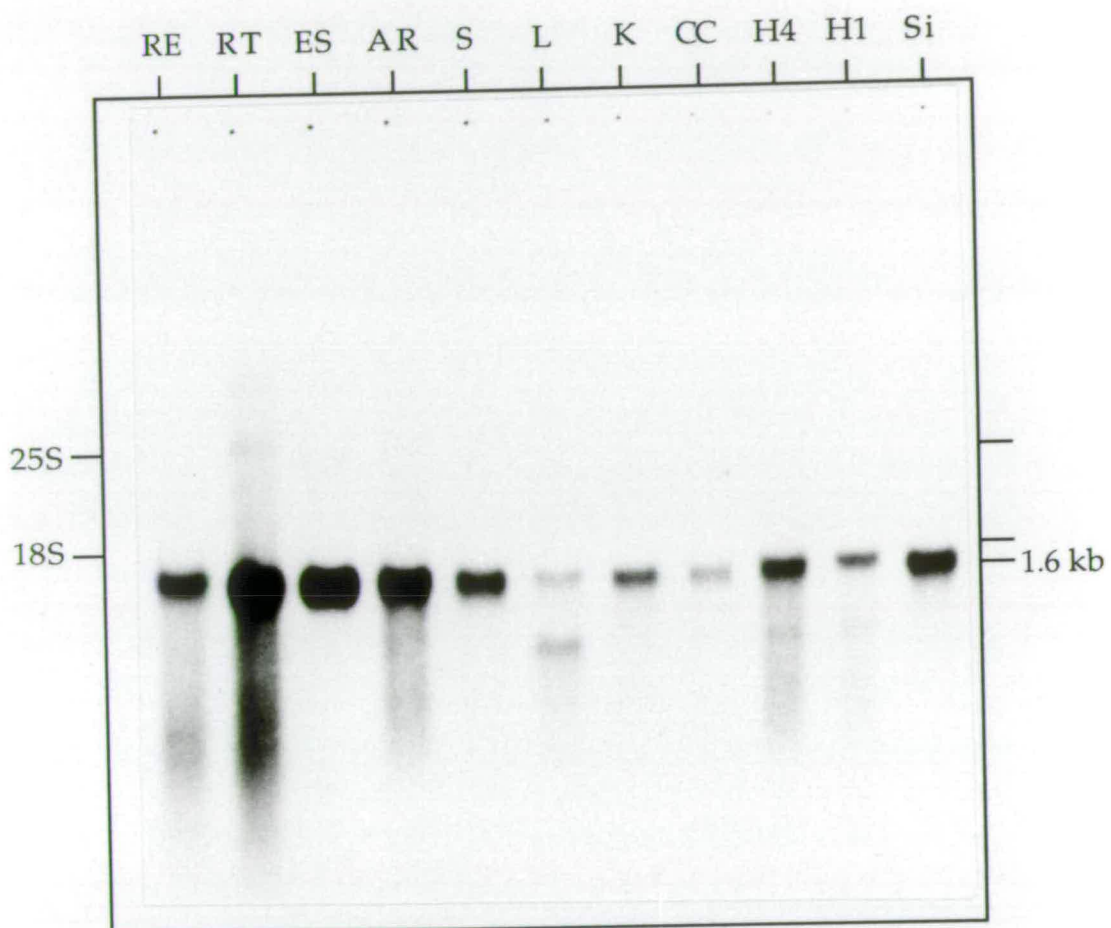
Northern blot analysis of different maize tissues with the universal *ANT* RNA probe derived from pMANT2 (using the T3 promoter and linearising the pMANT2 plasmid with PstI). 8 µg of etiolated seedling total RNA was used as a standard. The other samples were loaded so that the maize ribosomal bands were of equal intensity calculated by densitometry of the ethidium bromide stained bands (Appendix I). *ANT* transcript steady state levels were quantified for each tissue by densitometry as described in Appendix I. The ratios of the transcript levels between the various maize tissues are tabulated below:

RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
2.5	10	8.5	6.5	2.5	1	1.5	1	2.5	1.5	3.5

A key of the different tissues is presented below:

- RE root elongation zone
- RT root tip
- ES etiolated seedling
- AR adventitious root
- S stem
- L mature leaf
- K kernel (pre-pollination)
- CC centre of the cob
- H4 husk leaf 4 (inner)
- H1 husk leaf 1 (outer)
- Si pre-emergent silks

The markers are ribosomal RNAs from maize.



The highest *ANT* transcript levels were found in the non-photosynthetic vegetative tissues. These include the adventitious roots, the etiolated seedlings and the root tips. Mature leaves have the lowest steady state levels of all the tissues studied while the stem tissue, which is partially photosynthetic, has an intermediate *ANT* transcript level. These data suggest an inverse correlation between the photosynthetic competence of a tissue and the steady state level of *ANT* transcripts. This was confirmed by analysing *ANT* transcript levels in husk leaves. Husk leaves surround maize cobs and become progressively less photosynthetic from the outer leaf to the inner leaf (section 5.2). Comparison of *ANT* transcript levels between husk leaf 1 (outer) and husk leaf 4 (inner) reveals a higher amount of *ANT* transcript in husk leaf 4 (Figure 5.3). However, the above correlation cannot explain all observations since some non-photosynthetic tissues have low *ANT* transcript levels. These tissues include young kernels, the centre of the cob and the root elongation zone. A comparison of *ANT* transcript levels between the root tip and the root elongation zone reveals a difference. Since both regions are non-photosynthetic other factors, in addition to photosynthetic competence, must also regulate *ANT* transcript levels in a particular tissue. In this case root tip tissue includes meristematic cells, which are dividing and differentiating (section 1.3.1.2), whereas the elongation zone cells do not divide. Therefore, the higher *ANT* transcript level in root tips compared to the elongation zone correlates with the higher metabolic activity in root tips. The relatively high transcript levels observed in the pre-emergence silks may also be related to a high metabolic activity since they are growing rapidly and may require large amounts of energy to sustain the growth (Sheridan, 1982).

In all non-photosynthetic tissues mitochondria are the sole source of energy. Conversely, photosynthetic tissues have two sources of energy, mitochondrial oxidative phosphorylation and chloroplastic photophosphorylation. Thus, the reduction of *ANT* transcript levels in photosynthetic tissues may reflect the lower dependence on mitochondrial function. Similarly, tissues which have a lower metabolic rate will also utilise less energy and presumably have a lower dependence on mitochondrial function. Hence, they may also have a reduction in *ANT* transcript levels. If this hypothesis is correct it predicts that the metabolic activity of the cob centre and young kernels, prior to pollination, is

relatively low. A regulatory mechanism which monitors cellular energy levels and subsequently modulates *ANT* transcript levels has not been identified but may exist at the transcriptional or post-transcriptional level. Some putative regulatory mechanisms for the coarse control of expression of the genes involved in oxidative phosphorylation are discussed in section 1.4.2.

5.4.3 Are the two maize *ANT* genes differentially expressed?

Multiple copies of the *ANT* gene are present in both human and bovine genomes (section 1.6), and they are expressed independently in a tissue specific manner (Lunardi and Attardi, 1991). Differential expression is also common for individual members of yeast mitochondrial multi gene families such as isocytochrome *c* genes (Laz *et al.*, 1984) and cytochrome oxidase subunit V genes (Trueblood and Poyton, 1988). In order to investigate if maize *ANT* genes are differentially expressed gene specific probes for *ANT* genes were hybridised to northern blots of maize RNA extracted from different tissues and stages of plant development. A comparison of northern blots hybridised with either gene specific probes or a universal *ANT* probe is shown in Figure 5.4a. Each northern blot included a control filter with a serial dilution of pMANT1 and pMANT2 sense RNA (section 5.4.1.1 and Figure 5.4b). These control filters were used to:

- 1) Calculate the film response curve to known amounts of hybridised radioprobe (see Appendix I and section 5.4.2).
- 2) Quantify the specific activity of the probes to provide a comparison of signals from different northern blots.

After repeated attempts with *ANT1* specific probes derived from the 5' and 3' termini of pMANT1 the background levels were high while the intensity of the *ANT1* transcripts were low (Figure 5.4a). Consequently, little useful data were obtained from these northern blots. For the pMANT2 specific northern blots the ratio of the *ANT2* specific hybridisation signals between the various maize tissues (the transcript ratios) follow the same trend as the ratios calculated for the universal probe except the range is 3.5 fold compared to 10 fold (Figure 5.4a). Due to

Figure 5.4

A Northern blot analysis of RNA from various maize tissues using the universal *ANT*, *ANT1* specific and *ANT2* specific RNA probes. The universal *ANT* RNA probe was synthesised using the T3 promoter from pMANT2 which had been digested with the PstI endonuclease restriction enzyme (Figure 4.1 and 4.4). The *ANT1* and *ANT2* specific probes were complementary to the 3' end of the cDNA clones. They were synthesised using the SP6 and T3 promoters from pMANT1 and pMANT2 (linearised with Sau3A) respectively (Figure 4.1 and 4.4).

8 μ g of etiolated seedling total RNA was used as a standard. The other samples were loaded so that the maize ribosomal bands were of equal intensity calculated by densitometry of the ethidium bromide stained bands (Appendix I). *ANT* transcript steady state levels were quantified for each tissue by densitometry as described in Appendix I. The ratios of the transcript levels between the various maize tissues are tabulated (MANT2 specific = Maize *ANT2* specific probe; NS *ANT* = universal (non-specific) *ANT* probe).

A key of the different tissues is presented below:

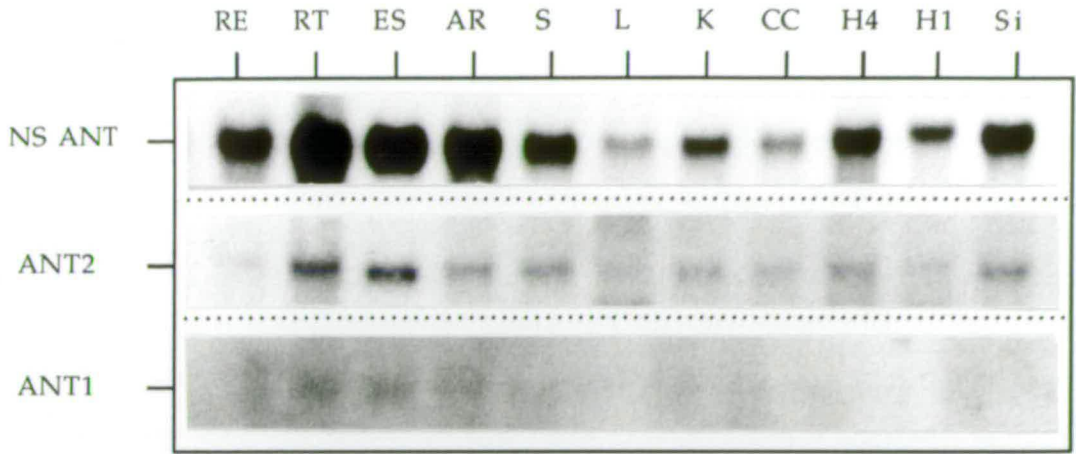
RE	root elongation zone	RT	root tip
ES	etiolated seedling	AR	adventitious root
S	stem	L	mature leaf
K	kernel (pre-pollination)	CC	centre of the cob
H4	husk leaf 4 (inner)	H1	husk leaf 1 (outer)
Si	pre-emergent silks		

B The control filters which were hybridised and autoradiographed with the respective northern blot filters above. Each lane has sense RNA corresponding to *ANT1* or *ANT2* which was synthesised from either pMANT1 or pMANT2. The NS *ANT* lane has pMANT2 sense RNA, the *ANT1* lane has pMANT1 sense RNA and the *ANT2* lane has pMANT2 sense RNA. PP
X

These hybridisation signals were used to quantify the film response curve and calculate the *ANT* transcript ratios between the various maize tissues above in A. The numbers either side of the figure are the relative concentrations of the sense RNA in each slot on the filter.

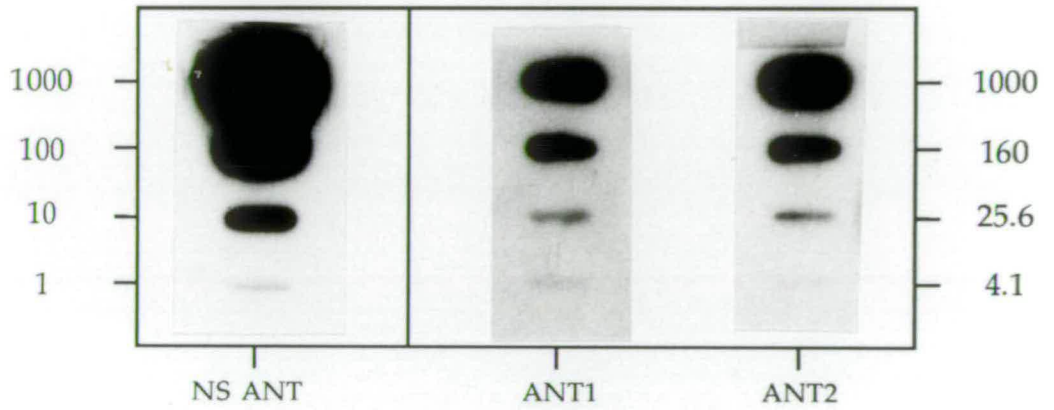
C Histogram comparison of the total *ANT* transcript ratios and the *ANT2* specific transcript ratios between the various maize tissues.

A

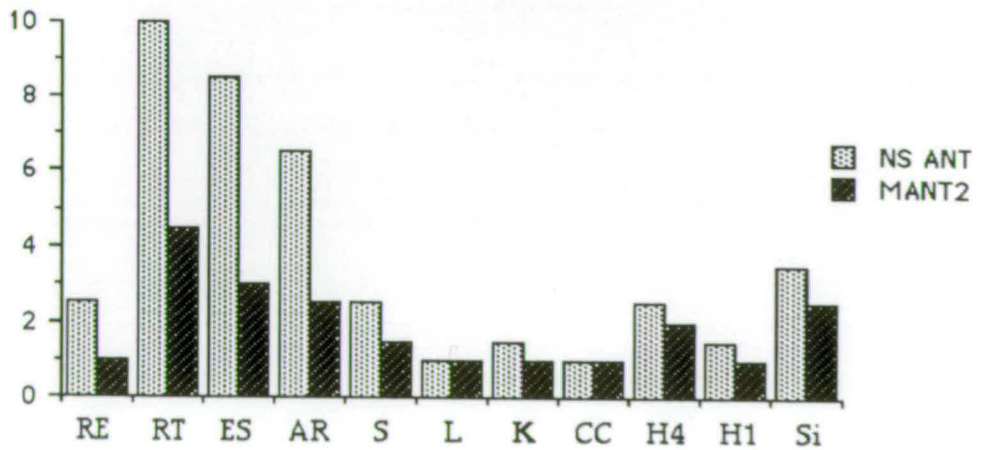


	RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
NS ANT	2.5	10	8.5	6.5	2.5	1	1.5	1	2.5	1.5	3.5
MANT2 Specific	1	4.5	3	2.5	1.5	1	1	1	2	0.95	2.5

B



C



the low range of the *ANT2* transcript ratio the predicted range of the *ANT1* transcript ratio must be greater than 10 fold to account for the range of the transcript ratios using the universal probe (10 fold).

Hypothetically, if *ANT2*'s contribution to the combined *ANT1/ANT2* transcript level is low the transcript ratio of *ANT1* will be approximately 10:1. If *ANT2*'s contribution to the combined transcript level is high, the transcript ratio of *ANT1* will be greater than 10:1. The maximum contribution by *ANT2* to the combined transcript level would occur if no *ANT1* is expressed in cob and leaf tissues (Figure 5.4c). In such a scenario *ANT1* transcripts would be more predominant than *ANT2* transcripts in the root tips, adventitious roots and etiolated seedlings. Consequently, these tissues probably have the most significant steady state levels of *ANT1* transcripts (Figure 5.4c). This is substantiated by the *ANT1* specific northern blot since a signal is detected in these tissues (Figure 5.4a).

5.4.4 Primer extension analysis of *ANT2* transcripts in light- and dark-grown maize leaves.

ANT2 has been shown to have two transcription initiation sites (section 4.9.1 and Figure 4.9). A role for the two different initiation sites is not known although they may be initiated at the different sites under different metabolic conditions. It has already been observed that *ANT* transcript levels are significantly higher in etiolated leaves compared to light-grown leaves (Figure 5.4a). In order to investigate whether significant increases in transcription from one or other of the two initiation sites is related to these growth conditions, primer extension analysis was carried out on green leaf tissue and etiolated leaf tissue from light- and dark-grown seedlings (Figure 5.5). There is no obvious difference in the usage of the initiation sites between these two growth conditions.

Figure 5.5

Primer extension analysis of RNA from light-grown leaves (L) and etiolated leaf tissue (ES) to determine whether significant increases in the transcription from one or other of the two initiation sites is related to growth conditions. The sizes of the gene specific primer extension products are indicated at the side and are derived from the sequencing reaction size markers in lanes 1-4. The size markers originate from a sequencing reaction using the *ANT2* genomic clone as a sequencing template and the *ANT2* specific oligonucleotide (Figure 4.7) as a primer. The same *ANT2* specific oligonucleotide is also used as a primer for the extension reactions in lanes 5-7.

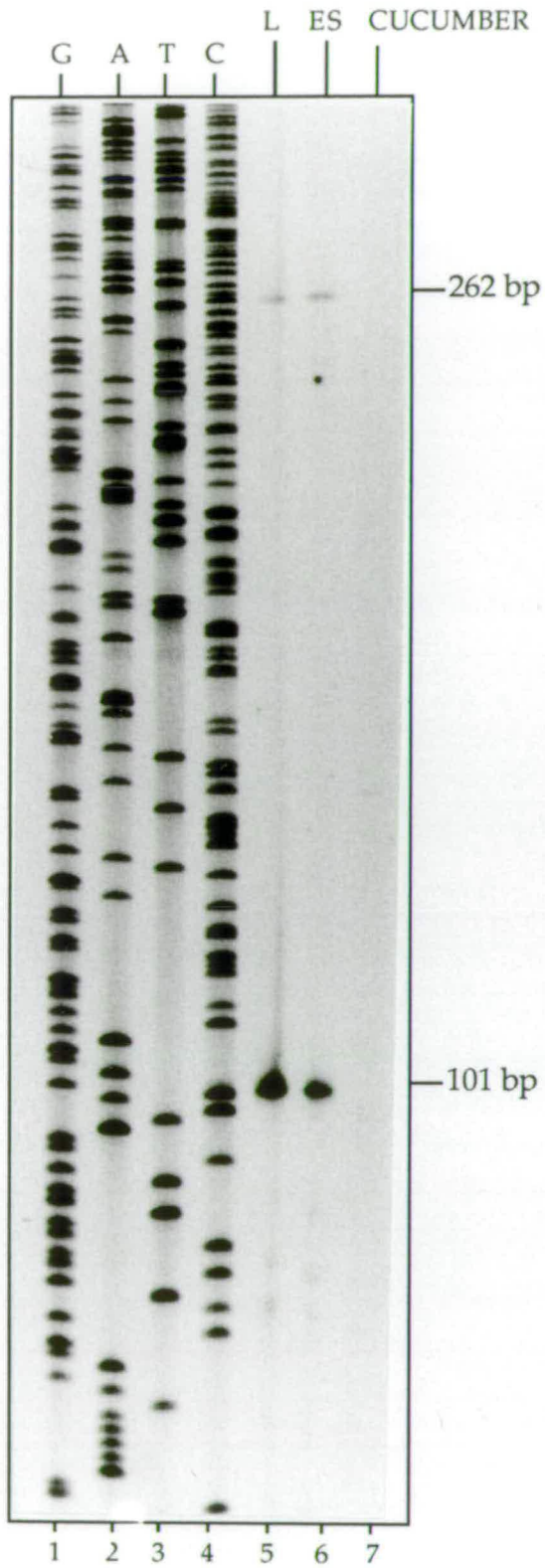
Lane 1-4 Marker sequencing reactions.

Lane 5 Primer extension with maize 3 -day-old dark-grown (etiolated) seedling RNA.

Lane 6 Primer extension with maize green leaf tissue.

Lane 7 Primer extension with cucumber 3-day-old cotyledon RNA.

C = cytosine, T = thymine, A = adenine, G = guanine.



5.5 ARE THE GENES ENCODING SUBUNITS OF MAIZE ATP SYNTHASE COORDINATELY EXPRESSED DURING DEVELOPMENT?

ATP synthase is a complex protein comprised of subunits encoded in both the nuclear and mitochondrial genomes (Vignais and Satre, 1984). As discussed in section 1.5, genes encoded in the two genomes may be coordinately expressed to maintain the correct subunit stoichiometry in the functional enzyme complex. Expression of ANT and ATP synthase genes may also be coordinated with other genes associated with oxidative phosphorylation. To investigate this proposal I analysed the expression of ATP synthase genes encoded in both genomes together with the ANT gene in various tissues of maize. Clones of maize ATP synthase subunits were available for two mitochondrial genes, the α subunit (Isaac *et al.*, 1985) and *ATP9* (Dewey *et al.*, 1985), and one nuclear gene, the β subunit (Winning *et al.*, 1990). The results of northern blot hybridisation using gene specific RNA probes are summarised in Figure 5.6.

5.5.1 EXPRESSION OF NUCLEAR GENES ENCODING SUBUNITS OF ATP SYNTHASE.

The ATP synthase β subunit has a similar steady state level to the ANT gene (Figure 5.6). This data suggests that in maize there may be coordinated expression of genes encoding proteins involved in oxidative phosphorylation. In humans regulatory sequences upstream of several OXPHOS genes have been identified (Evans and Scarpulla, 1989; Tomura *et al.*, 1990; Li *et al.*, 1990; section 1.5.2). Similar regulatory sequences may be responsible for the coordinated expression of maize ATP synthase and ANT genes.

5.5.2 EXPRESSION OF MITOCHONDRIAL GENES ENCODING SUBUNITS OF ATP SYNTHASE.

Analysis of RNA isolated from various maize tissues revealed a complex hybridisation pattern for both α subunit and *ATP9* genes (Figure 5.6). The variety of *ATP9* transcript sizes is due to complicated processing events occurring in the 5' and 3' flanking sequences (Dewey *et al.*, 1985). For α subunit it is not known if multiple initiation and termination events, or

Figure 5.6

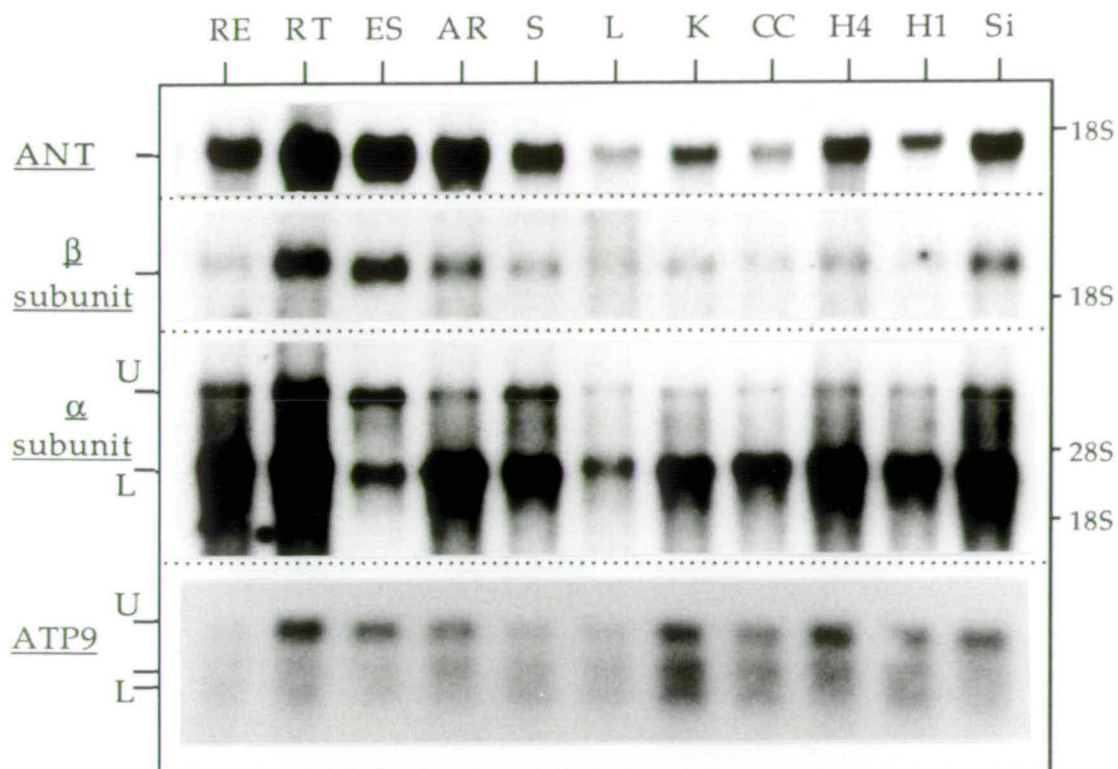
Northern blot analysis of RNA from various maize tissues using the universal ANT RNA probe (Figure 5.3) and RNA probes for three ATP synthase subunits, one encoded in the nucleus (β subunit (Winning *et al.*, 1990) and two encoded in the mitochondrial genome (*ATP9* (Dewey *et al.*, 1985) and α -subunit (Issac *et al.*, 1985)).

8 μ g of etiolated seedling total RNA was used as a standard. The other samples were loaded so that the maize ribosomal bands were of equal intensity calculated by densitometry of the ethidium bromide stained bands (Appendix I). *ANT* transcript steady state levels were quantified for each tissue by densitometry as described in Appendix I. The ratios of the transcript levels between the various maize tissues are tabulated below the northern blot data. For the mitochondrial genes the ratios of the transcript levels between the various tissues were calculated for the upper and lower hybridisation signals separately, as well as for the total (U + L).

A key of the different tissues is presented below:

RE	root elongation zone
RT	root tip
ES	etiolated seedling
AR	adventitious root
S	stem
L	mature leaf
K	kernel (pre-pollination)
CC	centre of the cob
H4	husk leaf 4 (inner)
H1	husk leaf 1 (outer)
Si	pre-emergent silks

The markers are ribosomal RNAs from maize.



	RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
NS ANT	2.5	10	8.5	6.5	2.5	1	1.5	1	2.5	1.5	3.5
β subunit	0.95	4	3	2	1	1	0.85	0.8	1	0.7	2
α subunit U	3.5	6	4	2.5	3.5	1	1.5	1.5	3	2	5
α subunit L	2.5	5	1	4.5	2.5	1	2	2.5	3	2	3.5
α subunit T	2.5	5	2	4	2.5	1	2	2	3	2	4
ATP9 U	0.65	4.5	2.5	3	1	1	3	2.5	3	1.5	2.5
ATP9 L	0.85	1.5	0.65	1.5	1	1	3	2	1.5	1.5	1
ATP9 T	0.8	2.5	1	2	1	1	3	2	2	1.5	1.5

differential processing accounts for the various transcript sizes (Isaac *et al.*, 1985). Two genes encode α subunit in maize and consequently, the transcripts may be gene specific.

The upper (U) and lower (L) hybridisation signals for ATP9 and α -subunit were quantified using densitometry (Appendix II and Figure 5.6). The upper and lower hybridisation signals for the respective ATP and α subunit genes were then combined and a transcript ratio was estimated between the various maize tissues. Neither α subunit or ATP9 genes show a similar pattern of expression compared to the β subunit and ANT nuclear genes. The steady state levels of mRNA for the ATP9 and α subunit genes are similar in all the tissues studied, except for leaf tissue, which gives a lower signal for both genes. These data suggest there is little, if any, coordination of expression between nuclear and mitochondrial genes encoding subunits of maize ATP synthase. Despite this conclusion it is intriguing that the upper transcript of α subunit, which contributes a lower proportion of the total, does show a similar pattern of expression compared to β subunit and ANT nuclear genes. The significance of this observation is hard to interpret.

Chomyn and Lai (1990) investigated the coordination of expression of nuclear and mitochondrial genes encoding subunits of mammalian NADH dehydrogenase (complex I). Their results also suggested that mitochondrial transcripts were maintained at a relatively constant level while nuclear transcript levels varied between different bovine tissues.

5.6 SUMMARY

ANT transcript steady state levels do vary in a tissue specific manner. The highest steady state transcript levels are found in tissues which exhibit mitochondrial oxidative phosphorylation and high metabolic rates. Tissues with lower metabolic rates or which are photosynthetically active contain lower transcript levels. Probes which differentiate between the two ANT genes were used to investigate the possibility of differential regulation. *ANT2* transcripts have been shown to have a 3.5 fold range whereas the range of *ANT1* transcripts are estimated to be greater than 10 fold. Another nuclear encoded gene, the β subunit of ATP synthase, has been shown to have a similar expression pattern to *ANT*. Whether the

expression of ANT and β -subunit nuclear genes is coordinated by regulatory elements common to both is not known. Mechanisms responsible for the regulation of *ANT* transcript levels and possible coordination of oxidative phosphorylation genes remain unidentified.

Expression analysis of two mitochondrial genes, *ATP9* and α subunit, both subunits of ATP synthase, reveals much less variation of transcript levels between the different tissues. This suggests there is little, if any, coordination at the level of transcription between nuclear and mitochondrial genes encoding subunits from the same protein complex.

CHAPTER 6

GENERAL DISCUSSION

6.1 SUMMARY OF RESULTS

Recent biochemical analysis of plant mitochondria has centred on ANT as a potential key regulatory protein of oxidative phosphorylation (section 1.4.1.1). As a consequence work in our laboratory was undertaken to clone ANT genes from maize. Prior to this thesis one *ANT* cDNA clone and two genomic clones had been isolated and partially characterised (Baker and Leaver, 1985; Baker, 1985; Bathgate *et al.*, 1989; section 3.2). The objective of the work described in this thesis was to use *ANT* gene specific probes to understand the molecular control and coordination of mitochondrial biogenesis in response to developmental and environmental signals.

The work presented describes further characterisation of the *ANT* genes and isolation of the second cDNA clone (Chapter 4). Steady state levels of *ANT* transcript have been analysed in different maize tissues and compared with other nuclear and mitochondrial genes encoding subunits of ATP synthase (Chapter 5). The principal achievements of the research presented in this thesis are summarised below:

- i) A cDNA clone, pMANT2, corresponding to the genomic clone *ANT2* has been isolated and sequenced (sections 4.4 and 4.5).
- ii) I have demonstrated that the start of transcription and translation were incorrectly identified by Bathgate *et al.* (1989).
 - a) The 5' end of the gene is much more complex than originally concluded. There are multiple transcription initiation sites and at least two introns in the 5' UTR (sections 4.8 and 4.9). The additional 5' exons and introns identified account for all the upstream genomic sequence which has been cloned to date (Figure 4.3). Consequently, neither the promoter or transcription initiation sites are located on the genomic clones.
 - b) The *ANT* open reading frame was found to have an additional 58 amino acids at the amino terminus (Sarah, 1991

and section 4.5.3) confirming that the cDNAs encode a presequence which is cleaved following import into mitochondria.

iii) The steady state levels of *ANT* transcript varied up to 10 fold between the tissues studied and a tentative correlation has been made between the level of transcripts and a tissue's demand for metabolic activity. Steady state levels were found to be lower in tissues with energy requirements partially satisfied by photophosphorylation or those with a low metabolic rate (section 5.4.2).

iv) Steady state levels of *ANT* and ATP synthase β subunit transcripts were similar in all maize tissues analysed (section 5.5). This observation may indicate that their expression is coordinated, which could be a consequence of both proteins being essential for oxidative phosphorylation. Conversely, there is no obvious coordination of transcript levels between mitochondrial (α subunit and *ATP9*) and nuclear (β subunit) genes that encode subunits of ATP synthase.

The following sections discuss the structure of the maize *ANT* gene in comparison to *ANT* genes from other species. The maize *ANT* genes have several novel features which will be discussed with regard to the expression of *ANT* during mitochondrial biogenesis. These include encoding an extended amino terminal sequence and a heterogenous 5' UTR which has at least two large introns separating untranslated exons. Proposals for future research to further our knowledge of mechanisms that regulate the expression of *ANT* are also discussed.

6.2 COMPARISON OF THE MAIZE *ANT* GENE STRUCTURE WITH *ANT* GENES FROM OTHER SPECIES

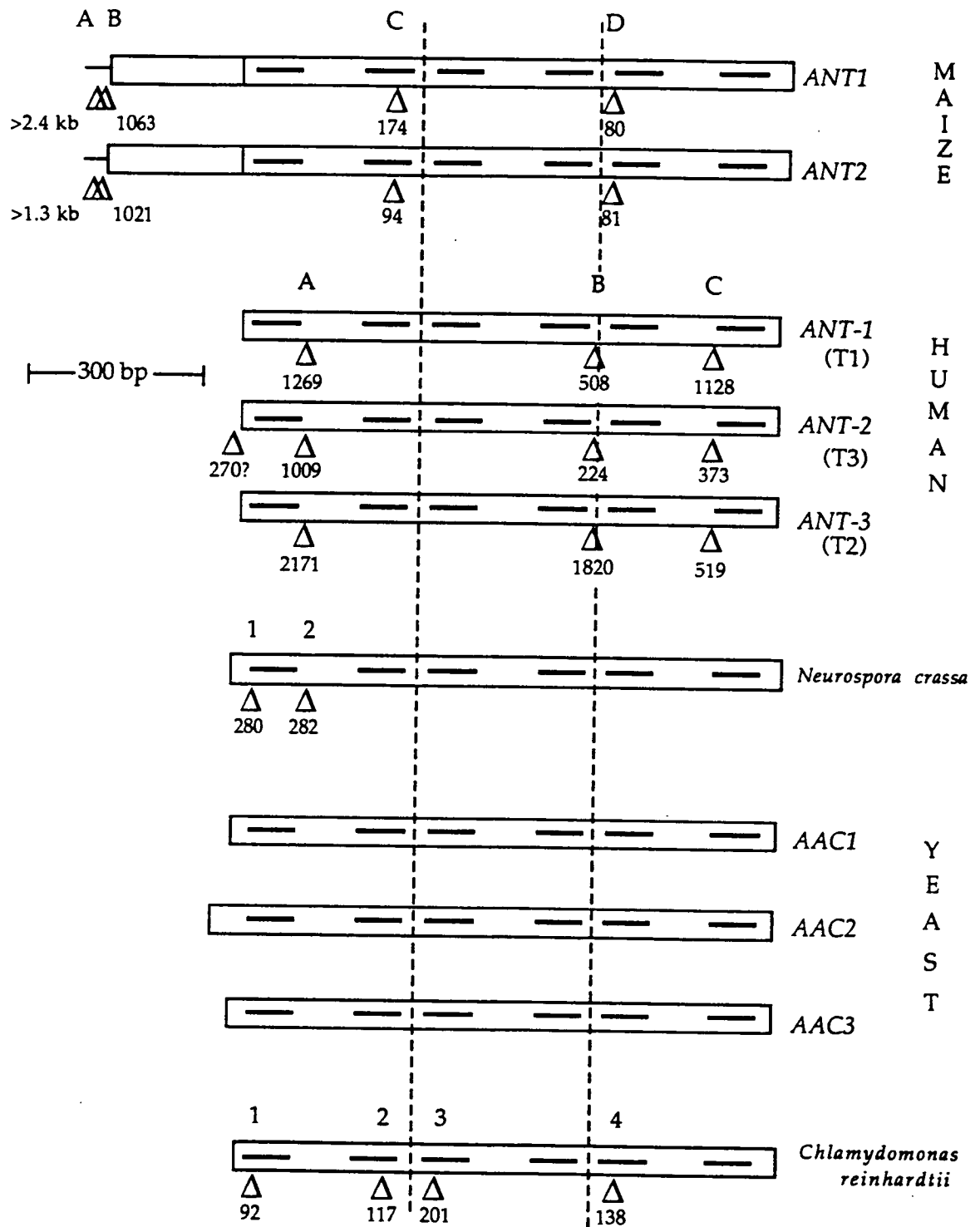
The *ANT* protein is a member of a family of well conserved inner mitochondrial membrane transport proteins which function as homo dimers. Other members of this family which share the same predicted structure but have different specificities are the uncoupling protein from mitochondria of brown fat, which is a proton transporter (Aquila *et al.*, 1985), the phosphate carrier (Runswick *et al.*, 1987) and the 2-oxoglutarate/malate carrier (Runswick *et al.*, 1990). The proposed

Figure 6.1

A comparison of the *ANT* genomic clones from various species such that the open reading frames are aligned. The open boxes represent the open reading frame and indicate the amino and carboxy termini encoded by each gene. The solid bars in each open reading frame represent the location of the predicted transmembrane α -helices. For a more detailed comparison of the predicted amino acid sequences from various species see figure 6.3.

The two dashed lines separate the three regions in the *ANT* open reading frame which show a significant similarity and are thought to have arisen from two gene duplication events (Saraste and Walker, 1982). The solid vertical lines at the 5' end of the maize *ANT* open reading frames indicate the location of the specific processing sites in these proteins.

The introns are represented by triangles (Δ) which indicate their location within the open reading frames. The intron sizes are indicated under the triangles in bp. Notation used to refer to the introns in the text are shown above each species group.



structure of these proteins is six transmembrane α -helices linked by loops outside the lipid bilayer (Saraste and Walker, 1982). Saraste and Walker (1982) have also described a 3 fold internal repeat of about 100 amino acids suggesting that these genes have evolved through two duplications of an ancestral gene which would have consisted of two transmembrane α -helical regions linked by a loop (for a review Klingenberg, 1988, Figure 6.1).

6.2.1 A comparison of the ANT gene introns between various species

ANT genes have now been cloned from a diverse range of species including mammals (Necklemann *et al.*, 1987; Li *et al.*, 1989; Houldsworth *et al.*, 1988; Powell *et al.*, 1989), fungi (Arends and Sebald, 1984), yeast (Adrian *et al.*, 1986; Kolarov *et al.*, 1990; Lawson and Douglas, 1988), algae (Hilgarth *et al.*, 1991; Sharpe and Day, 1992) and higher plants (Winning *et al.*, 1992, Winning *et al.*, 1991; Hashimoto *et al.*, 1992; Saint-Guily *et al.*, 1992). The gene structures of the genomic clones are presented in figure 6.1 such that the open reading frames are aligned. The location of the introns are conserved within an organism but there is little conservation between different species. The only conserved intron between two species is the intron C from maize and intron 2 from *Chlamydomonas* (Figure 6.1). The three human ANT introns B and possibly the two maize ANT introns D are situated in a location that may be considered a relic of earlier gene duplication events (Gilbert, 1978)(Figure 6.1). The other introns are all within the 100 amino acid tandem repeats and some interrupt the sequences which are predicted to be transmembrane α -helices. Such introns are unlikely to have had a role in gene duplication events and it is unclear if these introns have been acquired recently or have been lost by the other species. The maize ANT genes are unique in that they contain two introns within the 5' UTR although we have discovered that an intron may be located in the upstream region of the human ANT-2 gene. A 92 bp sequence upstream of the human fibroblast ANT gene (Ku *et al.*, 1990) is 48% similar to the MANT1 5' UTR (Figure 6.2). Ku *et al.* (1990) only used S1 nuclease protection to map the 5' terminus of the human ANT-2 gene and hence, primer extension analysis of their transcript may reveal human ANT-2 also contains an untranslated exon. Furthermore, analysis of the human ANT-2 genomic clone reveals a putative splice site which may represent an intron:exon boundary near the S1 nuclease site predicted to be the human ANT-2 transcript initiation site. The putative

Figure 6.2

Comparison of the maize *ANT* cDNA clone pMANT1 with the human *ANT-2* genomic clone (Ku *et al.*, 1990). Boxed nucleotides in the human sequence represent putative intron processing sites. The underlined ATG codons are the translational initiation codons in the two genes. The S1 nuclease protection analysis site identified by Ku *et al.*, (1990) is indicated by an open circle above the human genomic sequence. The open triangle above the pMANT1 sequence indicates the location of intron B in the maize gene *ANT1*.

```

1 .....COG CGCCTCTGAA AGTTGGGAGC CGCOGGGCOCG TCCGCTOCC
      |  |  ||  |  ||           |  |||  |  ||  |  |
1601 CTAGTCTGG TCTGCTCCA AGGGTTTTTG TCCAGGCACA TCGTGACCTC
      |  |  |||  ||  ||  ||  |  |||  |
44  TCTCGOCTC CCGTCCCAA GGOC..... 65
      |  |  |||  ||  ||  ||  |  |||  |
1651 ACCCTTCTC CCTCTCCGA GGCTCTCTC AGGCTCAGC GTTCAAGTCC
      |  |  |||  ||  ||  ||  |  |||  |
1701 CGGGTGTCT CTGGAOCCG CCTTGTCTT CGCOGGGTCA GGTGOOGAGG
1751 GGAGCACGGG CGGCGGGAG AGCAGTCCG GCOCGCOCTC CACGACTOCT
1801 CCTOCTGCGA GCTGGGCCAC TCAGGTCTGC CGCCAGCGC GCOGGGGOCC
1851 AGACCCCGOC CCGGCCCCGC CTCCGACGCC TGCOGCTCCA GCTCCGGCTC
66 ..... 65 .....AATAGT
      |  |  |||  ||  ||  ||  |  |||  |
1901 CCCTATATA AATCGGCAT TTGCTTCCGT CCGCCCGCA GGCGGAGT
      |  |  |||  ||  ||  ||  |  |||  |
74 TTTCAAGTGG CAATG 88 — Maize cDNA clone pMANT1
      ||  |
1951 CAAACGGTTC CCGGCCAGT CCGTCTCTGC AGCAGTCTGC CTCTCTTTC
2001 AACATGACAG ATGCC 2015 — Human genomic clone ANT-2

```

splice sites are highlighted in figure 6.2 as well as a pyrimidine-rich region which shows a high similarity with the pMANT1 sequence (Figure 6.5). The possible conservation of an intron in the 5' UTR between animals and plants may represent an important discovery. Putative roles for these introns will be discussed in section 6.4.

6.2.2 A comparison of the amino terminal sequence of ANT proteins between various species

A striking difference between the various ANT genes is the extended amino terminal sequence of the maize ANT genes (Figure 6.1 and Figure 6.3). Sequencing of the pMANT2 cDNA clone and resequencing of the *ANT2* genomic clone, presented in this thesis (section 4.3), confirmed the presence of an extended ANT open reading frame in maize which encoded the amino terminal sequence. Other plant ANT cDNA clones which have been recently isolated from *Solanum tuberosum* (potato), *Arabidopsis thaliana* and *Oriza sativa* (rice) (Winning *et al.*, 1992; Saint-Guily *et al.*, 1992; Hashimoto *et al.*, 1992) all encode an extended amino terminus supporting our observation in maize (Winning *et al.*, 1991) (Figure 6.3). In addition, an *ANT* cDNA has been cloned from the green algae, *Chlorella* which has an extended amino terminus (Hilgarth *et al.*, 1991). Winning *et al.*, 1992 have demonstrated that when the maize and potato cDNA clones are expressed *in vitro* the amino terminal extension is processed upon import into isolated mitochondria. This may be unique to plants since in *Neurospora* and yeast the ANT is imported without being processed, apparently using internal targeting information rather than the more usual mitochondrial targeting presequence (Pfanner *et al.*, 1987; Smagula and Douglas, 1988; Schatz, 1987). The amino terminal sequence of the ANT from higher plants may constitute a mitochondrial targeting sequence. *In vitro* import experiments with a maize ANT (97 amino acids of the amino terminus)-DHFR fusion protein into isolated mitochondria provides evidence that the ANT presequence can direct mitochondrial import (Winning *et al.*, 1992). Analysis of the predicted amino terminal sequences encoded by the plant ANT genes reveals a significant similarity for the first 30 residues and these residues may play a role in mitochondrial targeting (Figure 6.3). However, the majority of residues in this sequence are either hydrophobic or uncharged and are unlikely to form the typical amphiphilic α -helices associated with

Figure 6.3

An alignment of the predicted amino acid sequences of the mitochondrial ANT cDNA and genomic clones from various species. The alignment was generated using the Wisconsin computer program PILEUP.

The abbreviations and symbols used in this diagram are summarised below:

AAC1/2/3: *Saccharomyces cerevisiae* **Arab:** *Arabidopsis thaliana*

Pot: *Solanum tuberosum* **Bov1/2:** *Bos taurus* T1 and T2 genes

Rice: *Oriza sativa* **Chlor:** *Chlorella*

Chlamy: *Chlamydomonas reinhardtii* **Neur:** *Neurospora crassa*

Hant1/2/3: *Homo sapiens* ANT-1, ANT-2 and ANT-3 genes

Maize1/2: *Zea mays* ANT1 and ANT2 genes

Sources for the ANT amino acid sequences presented in this comparison are referenced in table 6.1.

* A star beneath a column of amino acid residues indicates they are conserved in all the sequences.

Δ A triangle beneath a column of amino acid residues indicates they are not conserved but do have similar chemical properties in all the sequences.

|----| transmembrane domain

§ Indicates the amino terminus of the mature ANT proteins in maize (—AEKGG) and potato (AP—EKG—). The first amino acid residue in each case has been underlined. These sequences were identified by amino terminal sequencing of ANT proteins purified from maize and potato mitochondria respectively and thus indicate the location of the specific processing site (Winning *et al.*, 1992).

150	AVAKTASPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	AAC2
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	AAC3
	ALAKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	AAC1
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Arab
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Pot
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Matzel
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Matzel
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Rice
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Chlor
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Chlamy
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Neur
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Boz2
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Hant1
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Boz1
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Hant3
	AVSKTAAPI ERVKLIÖNÖ DE. MIKÖGT	DEKRAAGIÖD	CFKRTAÖEG	Hant2

100	AÖVKTRIPPA	PARKESNFTL	IDFTMGVSA	AAC2
	MSSN	AK.	INFTMGVSA	AAC3
	MSSD	AK.	INFTMGVSA	AAC1
	MSSD	AK.	INFTMGVSA	Arab
	MSSD	AK.	INFTMGVSA	Pot
	MSSD	AK.	INFTMGVSA	Matzel
	MSSD	AK.	INFTMGVSA	Matzel
	MSSD	AK.	INFTMGVSA	Rice
	MSSD	AK.	INFTMGVSA	Chlor
	MSSD	AK.	INFTMGVSA	Chlamy
	MSSD	AK.	INFTMGVSA	Neur
	MSSD	AK.	INFTMGVSA	Boz2
	MSSD	AK.	INFTMGVSA	Hant1
	MSSD	AK.	INFTMGVSA	Boz1
	MSSD	AK.	INFTMGVSA	Hant3
	MSSD	AK.	INFTMGVSA	Hant2

50	HÖVÖPPTI	AÖKRAÖF	M	RSSVSÖVÖ	..	VGÖRPSM	YÖRHATYAN	Arab
	MADN	ÖHPTV	YÖKVASÖMHT	SSSISÖDVHA	RYGGIÖRPAT	SÖRFFPYAN	Pot	
	MADN	ÖHPTV	YÖKVASÖMHT	SSSISÖDVHA	RYGGIÖRPAT	SÖRFFPYAN	Matzel	
	MADN	ÖHPTV	YÖKVASÖMHT	SSSISÖDVHA	RYGGIÖRPAT	SÖRFFPYAN	Matzel	
	MADN	ÖHPTV	YÖKVASÖMHT	SSSISÖDVHA	RYGGIÖRPAT	SÖRFFPYAN	Rice	
	MADN	ÖHPTV	YÖKVASÖMHT	SSSISÖDVHA	RYGGIÖRPAT	SÖRFFPYAN	Chlor	

AAC2	SFLLGWVVT	GASTCSYPLD	TVRRRMMMS	...	GQAVKYD	GAFDCLRKTIV
AAC3	SFLLGWVVT	GASTCSYPLD	TVRRRMMMS	...	GQAVKYN	GAIDCLKKTIV
AAC1	SFLLGWVITM	GASTASYPLD	TVRRRMMMS	...	GQTIKYD	GALDCLRKTIV
Arab	SFALGWITN	GAGLASYPID	TVRRRMMMS	..	NEAVKYK	SSLDAFQOIL
Pot	SFALGWLITN	GAGLASYPID	TVRRRMMMS	..	GEAVKYK	SSFDAFNQOIL
Maize1	SFALGWLITN	GAGLASYPID	TVRRRMMMS	..	GEAVKYK	SSLDAFQOIL
Maize2	SFALGWLITN	GAGLASYPID	TVRRRMMMS	..	GEAVKYK	SSLDAFQOIL
Rice	SFALGWLITN	GAGLASYPID	TVRRRMMMS	..	GEAVKYK	SSMDAFSQOIL
Chlor	KWAQAQAVTA	GAGVLSYFPD	TVRRRLMQS	...	GGERQYN	GTIDCWRKVA
Chlamy	AFLLGWGITI	GAGLASYPID	TIRRRMMMS	...	GSAVKYN	SSFHCFQEIIV
Neur	SFALGWCVTT	AAGLASYPID	TIRRRMMMS	...	GEAVKYK	SSFDAASQIV
Bov2	SWMIAQIVTA	VAGLVSYFPD	TVRRRMMMS	GRKGADIMYT	GTVDCWRKIA	
Hant1	SWMIAQSVTA	VAGLVSYFPD	TVRRRMMMS	GRKGADIMYT	GTVDCWRKIA	
Bov1	SWMIAQIVTA	VAGVSYFPD	TVRRRMMMS	GRKGADIMYK	GTVDCWRKIL	
Hant3	SWMIAQIVTA	VAGVSYFPD	TVRRRMMMS	GRKGADIMYT	GTVDCWRKIF	
Hant2	SWMIAQIVTA	VAGLTSYFPD	TVRRRMMMS	GRKGTIDIMYT	GTIDCWRKIA	
	Δ Δ Δ*	* *** *	*Δ***Δ** *	*ΔΔ	*	Δ
	--v-----					

AAC2	AAEGVGSFLK	GCGANILRGV	AGAGVISMYD	QLQMLFGKK	FK.....
AAC3	ASEGVSFLK	GCGANILRSV	AGAGVISMYD	QLQMLFGKK	FK.....
AAC1	QKEGAYSFLK	GCGANIFRGV	AAAGVISLYD	QLQLIMFGKK	FK.....
Arab	KNEGAKSLFK	GAGANILRAV	AGAGVLSGYD	KLTLIVFGKK	YGSGBA.
Pot	KNEGPKSLFK	GAGANVLRV	AGAGVLAGYD	KLQVIVFGKK	YGSGBG.
Maize1	KKEGPKSLFK	GAGANILRAI	AGAGVLSGYD	QLQILFFGKK	YGSGBA.
Maize2	KKEGPKSLFK	GAGANILRAI	AGAGVLSGYD	QLQILFFGKK	YGSGBA.
Rice	KNEGAKSLFK	GAGANILRAI	AGAGVLSGYD	QLQILFFGKK	YGSGBA.
Chlor	QOEGMKAFFK	GAWSNVLGA	GGAFVLVLYD	EIKKFVNPNA	VSSASE.
Chlamy	KNEGKMSLFLK	GAGANILRAV	AGAGVLAGYD	QLQVILLGKK	YGSGBA.
Neur	AKEGVKSLFK	GAGANILRGV	AGAGVLSIYD	QLQVLLFGKA	FKGGSG.
Bov2	KDEGPKAFFK	GAWSNVLGRM	GGAFVLVLYD	EIKKFV....
Hant1	KDEGAKAFFK	GAWSNVLGRM	GGAFVLVLYD	EIKKYV....
Bov1	KDEGGKAFFK	GAWSNVLGRM	GGAFVLVLYD	ELKKVI....
Hant3	RDEGGKAFFK	GAWSNVLGRM	GGAFVLVLYD	ELKKVI....
Hant2	RDEGGKAFFK	GAWSNVLGRM	GGAFVLVLYD	EIKKYT....
	**	** *	*Δ *	ΔΔ* *Δ	** Δ
	-----VI-----				

mitochondrial targeting sequences (von Heijne, 1986; von Heijne *et al.*, 1989). A possible role for these presequences in the plant ANT proteins and their likely origin will be discussed further in section 6.3.

6.2.3 A comparison of the ANT protein sequences between various species

The ANT gene sequences 3' of the maize processing site are well conserved in all the species, particularly the predicted transmembrane regions which occur in similar locations (Figure 6.3). In most cases the percentage identity of ANT genes within a species is significantly greater than that between species (Table 6.1). This suggests that the ANT gene duplication events have occurred after divergence of the maize, human and yeast. Two bovine ANT cDNA clones show a better similarity with the human ANT-1 and ANT-3 clones than with each other, indicating that in this case the duplication occurred before the human and bovine species had diverged (Cozens *et al.*, 1989)(Table 6.1). The ANT gene from *Chlorella* is unusual since it has a high similarity with the vertebrate ANT genes despite being more closely related to photosynthetic organisms (Beckett *et al.*, 1971; Palmer *et al.*, 1988). This may indicate the limitation of sequence comparisons from one gene alone to determine relationships between species.

6.3 ORIGIN OF THE PRESEQUENCE

Higher plants are unusual in that their ANT proteins have long amino terminal sequences (section 6.2). In addition the amino terminal sequences from maize and potato ANT proteins expressed *in vitro* have been shown to be processed upon import into isolated mitochondria (Purdue, 1988; Sarah, 1991; Winning *et al.*, 1992). To date, presequences have only been identified in ANT proteins from photosynthetic species, hence, a speculative role for the presequence is to prevent missorting to the chloroplast (Sarah, 1991; Winning *et al.*, 1992).

Previous work by Huang *et al.* (1990) demonstrated that a fusion protein of a yeast mitochondrial presequence (yeast precursor to cytochrome oxidase subunit Va) and CAT reporter gene, is targeted to both chloroplasts and mitochondria in transgenic tobacco. Similar missorting of ANT in

Table 6.1

A comparison of the ANT proteins from various species. The numbers represent the percentage identity between the amino acid residues between the amino terminus of the maize and potato mature ANT proteins (Figure 6.3) and the carboxy terminus. Consequently, the precursor sequences characteristic of higher plants have not been considered for this comparison. The identities which are in 'outline' style indicate comparisons of closely related species or ANT genes from the same species.

Key of the species compared in table 6.1

Dicotyledonous plants

At: *Arabidopsis thaliana* (Saint-Guilly *et al.*, 1992)

St: *Solanum tuberosum* (potato) (Winning *et al.* 1992)

Monocotyledonous plants

Zm1/2: *Zea mays* (maize) ANT genes *ANT1* and *ANT2* (Winning *et al.*, 1991)

Os: *Oriza sativa* (rice) (Hashimoto *et al.*, 1992)

Nc: *Neurospora crassa* (Arends and Sebald, 1984)

Sc1/2/3: *Saccharomyces cerevisiae* (yeast) ANT genes *AAC1*, *AAC2* and *AAC3* (Adrian *et al.*, 1986; Lawson and Douglas, 1988; Kolarov *et al.*, 1990)

Algae

Cr: *Chlamydomonas reinhardtii* (Sharpe and Day, 1992)

Ck: *Chlorella kessleri* (Hilgarth *et al.*, 1991)

Vertebrate

Bt1/2: *Bos taurus* (bovine) ANT genes *T1* and *T2* (Powell *et al.*, 1989)

Hs1/2/3: *Homo sapiens* (human) ANT genes *ANT-1*, *ANT-2* and *ANT-3*. (Necklemann *et al.*, 1987; Li *et al.*, 1989; Houldsworth *et al.*, 1988)

	Bt1	Bt2	Hs1	Hs2	Hs3	Ck	Cr	Sc1	Sc2	Sc3	Nc	Zm1	Zm2	Os	At
St	54	55	55	47	46	51	80	65	72	69	74	88	88	90	91
At	54	54	56	55	53	50	79	68	71	71	70	89	90	91	
Os	55	53	56	55	53	49	81	65	72	72	75	95	96		
Zm2	54	54	48	47	46	50	80	66	72	71	76	99			
Zm1	54	54	47	47	46	50	80	66	71	70	75				
Nc	46	44	45	46	45	50	76	66	74	72					
Sc3	55	52	52	55	55	49	71	75	90						
Sc2	54	53	54	55	54	49	72	77							
Sc1	41	45	50	46	40	45	63								
Cr	54	54	57	55	54	49									
Ck	66	64	65	66	66										
Hs3	98	89	88	92											
Hs2	93	90	89												
Hs1	88	95													
Bt2	89														

primitive photosynthetic species may have led to selective pressure to evolve a more specific targeting mechanism. Recently *ANT* has been cloned from two primitive green algae, *Chlorella* (Hilgarth *et al.*, 1991) and *Chlamydomonas* (Sharpe and Day, 1992). Both are classified as Chlorophytes and are distantly related to higher plants (Beckett *et al.*, 1971; Palmer *et al.*, 1988; Bremer, 1985). A comparison of the higher plant and Chlorophyte predicted amino terminal protein *ANT* sequences was performed using the computer program PILEUP (Figure 6.3). This reveals that *Chlorella* has an extended open reading frame which is 31 amino acids longer than the mature maize and potato *ANT* proteins, while *Chlamydomonas* has no additional sequence. The maize and potato presequences have a similar amino acid sequence whereas the *Chlorella* presequence has no similarity and is shorter than the higher plant presequence (31 amino acids compared to 77 amino acids in maize and 76 amino acids in potato)(Figure 6.3). Whether the amino terminal sequence of *Chlorella* is processed upon import or has a similar function to the higher plant presequences cannot be predicted.

The fact that *Chlorella* and *Chlamydomonas* are closely related (Becket *et al.* 1971; Palmer *et al.*, 1988) implies that the *Chlorella* presequence has evolved relatively recently. This along with the observation that the higher plant and *Chlorella* presequences are not similar (Figure 6.3), implies that the presequence from higher plants and the presequence from *Chlorella* are the products of convergent evolution. In both cases the presequence may have evolved by mutations upstream of the mature protein coding sequence generating alternative ATG codons. A comparison of the nucleotide sequence which encodes a region of the *Chlorella* presequence and 72 nucleotides 5' of the *Chlamydomonas* ATG codon reveals a similarity of 46% (Figure 6.4a). This similarity would be expected between closely related species if a mutation had generated an alternative 5' ATG codon to form an extended open reading frame.

The absence of a presequence in *Chlamydomonas* is an indication that the presequence may have evolved since the endosymbiotic event which led to the ancestor of plants and algae. To determine an approximate timing for the evolution of an *ANT* presequence in higher plants, and provide further evidence to confirm the convergent evolution hypothesis, the

A

```

Chlorella cDNA sequence          CGGAGTGCCTCTTGG 15
                                | | | | |
Chlamydomonas ANT genomic sequence TGAAGAGCCCTCAGT 100

16 CTGCCTCTGCTTCTCTCTACTCGCTTGGGGGAGGAGAAGTCGCAGCCAAC 65
   | | | | | | | | | | | | | | | | | | | | | | | | | | |
101 CAGCCTCAAC.TCGCCCCATTCCCAGCGGTGTTTGCCCATACCACTTCCC 149

66 ATGCTGTCCTCGGCACTGTATCAGCAGGCAGGCCTGAGCGGCCTCCTGCG 115
   | | | | | | | | | | | | | | | | | | | | | | | | | | |
150 CTCTGGCGCGTCGTAGCGTCGTTGTCCTAGTGTAAGCGGCAGGAGCCC 199

116 CGCGTCCGCTATGGGCCCAGACACCCTTCATCGCCTCGCCAAAGGAGA 165
    | | | | | | | | | | | | | | | | | | | | | | | | | | |
200 AGCGTCTCCTCTCCATCTCGGTATAACCCTCGCCCGTCCCGGAAAGGGTC 249

166 CCCAGGCC.....GACCCGATGGCCTTTGTTAAGGATCTGCTGGCT 206
    | | | | | | | | | | | | | | | | | | | | | | | | | | |
250 CCGACAACATGGCCAAGGAGGAGAAGAAGTTCATGGTGGACTTCTGGCC 299
  
```

B

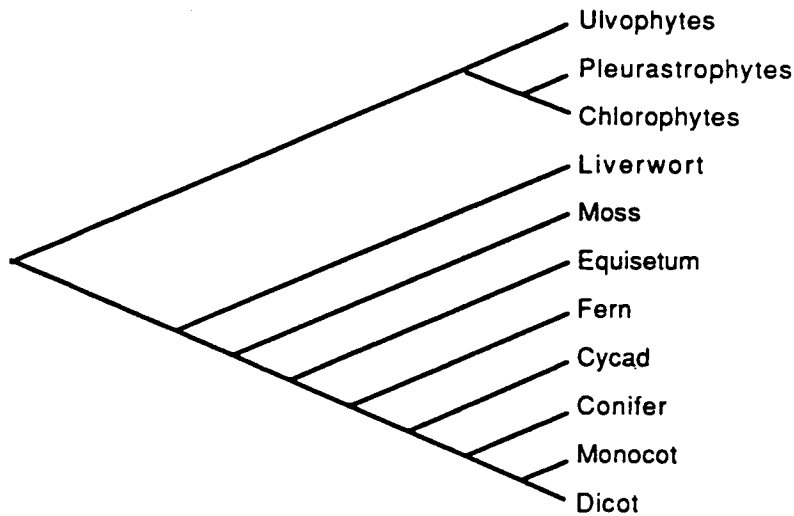


Figure 6.4

A Sequence comparison of the *Chlorella* ANT cDNA clone (Hilgarth *et al.*, 1991) with the *Chlamydomonas* ANT genomic clone (Sharpe and Day, 1992). The ATG translational initiation codons are underlined.

B Cladogram of photosynthetic species based on Bremer (1985). This indicates that the Chlorophytes, of which *Chlorella* and *Chlamydomonas* are members (Beckett *et al.*, 1971), are distantly related to higher plants.

ANT gene must be cloned from other photosynthetic species which are more closely related to higher plants (Figure 6.4b).

6.4 THE 5' GENE STRUCTURE OF THE MAIZE ANT GENES: ITS PUTATIVE ROLE IN THE REGULATION OF GENE EXPRESSION.

The work presented in this thesis demonstrates that the 5' gene structure of the maize *ANT* is unusual since both genes have at least two large introns in the 5' UTR and hence, at least two untranslated exons. Also, the length of the 5' UTRs vary considerably due to multiple initiation sites. *ANT2* has two distinct transcript initiation sites while *ANT1* has eight. Intron A could be in excess of 2.4 kb while intron B is 1063 bp and 1021 bp for *ANT1* and *ANT2* respectively (See section 4.8 and 4.9). These introns are much longer than other known plant introns, the average size being 249 bp (Hawkins, 1988). Also, their location in the 5' UTR is unusual since plant introns generally separate protein domains (Hawkins, 1988). However, this example is not unique and other genes which have large introns in their 5' UTRs include actin genes (McElroy *et al.*, 1990; Pearson and Meagher, 1990), maize *Shrunken-1* (Werr *et al.*, 1985), and soyabean translation elongation factor eEF-1a (Aquilari *et al.*, 1991). The following sections will discuss these 5' structural features and their possible roles in the regulation of *ANT* expression.

6.4.1 The role of the untranslated 5' exons

The significance of the presence of at least two untranslated exons in the 5' end of the maize *ANT* genes is not currently known. Neither is the significance of multiple initiation sites which generate 5' UTRs ranging from 125-230 bp for *ANT1* and 160 bp or 320 bp for *ANT2* (section 4.9.1). A study of 79 plant genes determined that the average size for a plant 5' UTR is between 40-80 nucleotides within a range of 9 to 193 nucleotides (Joshi, 1987a). Consequently, the 5' UTR from maize *ANT* is large compared with most other plant leader sequences and may have a functional role in *ANT* expression.

6.4.1.1 Is the 5' UTR of maize *ANT* conserved between species?

It is likely that functional sequences in the 5' UTR from the same gene would be conserved between species as is the case with the hairpin loop in the 5' UTR of ferritin mRNA (Leibold and Munro, 1988). The introns in the 5' UTR and the unusually long 5' UTR in maize *ANT* genes are not features conserved in *ANT* genes from other species and may represent plant specific structures. One exception to this is the putative intron in the 5' UTR of the human *ANT-2* gene discussed in section 6.2. The putative intron and a conserved pyrimidine-rich region are summarised in figure 6.2 which shows a high similarity with the pMANT1 untranslated leader sequence (Figure 6.5). The pyrimidine-rich sequence is also present in the pMANT2 cDNA, the untranslated exon of maize *Shrunken-1* and a putative untranslated sequence in the human *ANT-2* gene. A possible function is discussed in section 6.4.1.4.

6.4.1.2 A role for the 5' UTR in translational control?

A number of workers have shown that the 5' UTR of certain genes plays a role in regulating translation and hence controlling the levels of the corresponding protein (Gruissem, 1989 and Fox, 1986). Jobling and Gehrke (1987) have shown that mRNAs containing little or no 5' structure are normally translated with high efficiency. Secondary structure may inhibit translation of the mRNA by preventing the migration or initiation of the ribosomal initiation complex (Kozak, 1989). Common secondary structures are hairpin loops (ferritin mRNA (Leibold and Munro, 1988)) or pseudoknots (α operon of *Escherichia coli* (Tang and Draper, 1989)) which are often stabilised by specific proteins. The 5' UTRs of maize *ANT* transcripts, sequenced from the cDNA clones, have a high proportion of G/C bases (Figure 4.1) as does the sequence obtained further upstream of *ANT2* transcripts by RNA sequencing (Figure 4.12). This may indicate a high degree of secondary structure which could potentially be important for translational control.

6.4.1.3 A role for 5' end heterogeneity in maize *ANT* transcripts?

A role for the multiple initiation sites has been discussed in section 4.10. It was suggested that one of the *ANT* genes may be expressed in all tissues

with little variation (a 'housekeeping' role for *ANT1*) while the other was more highly regulated (*ANT2*). Preliminary data on differential expression of the two ANT genes in maize suggests that *ANT1* exhibits the greatest range of steady state transcript levels (section 5.4.3) which does not support the above hypothesis. Another plausible interpretation for the 5' end heterogeneity is alternative splicing in intron A. Examples of alternative splicing have been observed for some vertebrate genes with introns in the 5' UTR (Deák *et al.*, 1991; Luo *et al.*, 1989; Reynolds *et al.*, 1985). Alternative splicing produces transcripts which have variable UTRs resulting in untranslated leader sequences with different secondary structures. No function has been identified for this heterogeneity but a role in cell type- and stage-specific expression has been postulated by Deák *et al.* (1991). RNA sequencing of the *ANT2* transcripts (Figure 4.12) produced a sequence which represented the *ANT2* mRNA untranslated leader. If alternative splicing was a feature of *ANT2* transcripts two superimposed sequences would be expected. There is only one sequence which suggests that there is no alternative splicing in the *ANT* 5' UTR, although, it cannot be discounted that the second sequence corresponding to the largest *ANT2* specific primer extension product may be too faint to visualise.

6.4.1.4 Are there promoter elements in the *ANT* 5' UTR?

The untranslated exon from the maize gene *Shrunken-1* can increase the expression of a reporter gene by up to 10 fold in a transient expression assay using maize protoplasts (Maas *et al.*, 1991). Maas *et al.* (1991) have proposed that a novel type of RNA polymerase II promoter element may be present in this region since the exon is position dependent and apparently has its effect in conjunction with either the octopine synthase enhancer (*ocs* palindrome) or the cauliflower mosaic virus (CaMV) enhancer sequence (Tokuhisa *et al.*, 1990; Fang *et al.*, 1989). Since these enhancers are reported to interact with different transcription factors the putative nuclear protein recognising sequences in the untranslated leader of *Shrunken-1* can interact with a variety of different transcription factors. These characteristics are similar to a RNA polymerase III promoter element, located downstream of the 5S RNA genes of *Xenopus laevis*, which binds TFIIA and TFIIB (Geiduschek and Tocchini-Valentini, 1988). A pyrimidine-rich sequence in the *Shrunken-1* untranslated exon has

been postulated to have a role in binding nuclear factors (Maas *et al.*, 1990). It is interesting that a similar pyrimidine sequence is also present in the maize *ANT* untranslated exons 2 (Figure 6.5), although current evidence discussed in Chapter 4 indicates that the promoter region may be more than 2.4 kb upstream of maize *ANT* exon 2 (Figure 4.3). A role for the pyrimidine-rich sequence involving an interaction with the transcription complex is hard to envisage if the promoter region is several kb further upstream. It is not known whether this sequence has a function in the untranslated exon in maize *ANT*.

6.4.2 Possible roles for the 5' UTR introns in the maize *ANT* genes

Several functions are associated with intron sequences. These include exon shuffling (Gilbert, 1978; 1985), which is hypothesised to have a role in generating genetic diversity or new proteins. Alternative splicing of introns causes the production of diverse transcripts from a single gene (Smith *et al.*, 1989). This mechanism may produce heterogeneous transcripts which encode different proteins (Smith *et al.*, 1989) or have different regulatory characteristics (Deák *et al.*, 1991). Additionally, there are now reports of introns with a functional role in gene expression (Callis *et al.*, 1987). The role of the introns in the *ANT* 5' UTR have not been investigated experimentally but their large size and unusual location, separating untranslated exons, suggests that they may have a specific function.

6.4.2.1 Do the introns in the maize *ANT* genes 5' UTRs separate functionally distinct domains?

As discussed above 5' UTRs may be associated with translational (section 6.4.1.2 and 6.4.1.3) or transcriptional (section 6.4.1.4) control, hence, their sequences would be an important component of a gene. Gilbert (1978; 1985) has described a mechanism where sequences corresponding to different protein domains may be combined to form genes encoding new proteins. He suggests that as a consequence of this 'exon shuffling' the various domains encoded in a gene may be delimited by intron sequences. It is noticeable that many of the introns which separate 5' UTRs from coding sequence are within 10-26 nucleotides of the ATG initiation codon (Pearson and Meagher, 1990; Werr *et al.*, 1985; Deák *et al.*, 1991; Aquilar *et al.*, 1991). Similarly, introns B in the 5' UTR of maize *ANT* genes are

within 20 bp of the ATG initiation codon (Figure 4.7). Consequently, it is possible that the 5' UTRs have become associated with these genes by an 'exon shuffling' mechanism.

6.4.2.2 Introns in some genes appear to play a role in normal gene expression

Several genes have been shown to contain introns which appear to play a role in normal gene expression. Examples are the maize *Shrunken-1* (Maas *et al.*, 1991), rice actin 1 gene (McElroy *et al.*, 1990) and maize alcohol dehydrogenase (Callis *et al.*, 1987). The role of the introns has been investigated by analysing the transient expression of chimaeric genes which include these introns. A variety of chimaeric genes have been investigated including different promoters (endogenous and viral) and genes (endogenous and CAT and GUS reporter genes). Removal of the introns results in a much lower transcript steady state level while their insertion into the transcription unit of a chimaeric gene enhances its expression by up to 100 fold (Maas *et al.*, 1991; Callis *et al.*, 1987; Vasil *et al.*, 1989). Callis *et al.* (1987) determined that the optimal location for a maize alcohol dehydrogenase intron, resulting in high expression of a chimaeric gene, is near the 5' end of the nascent mRNA. Likewise, introns known to be important for the normal expression in endogenous genes are often localised at the 5' end of the nascent mRNA. In the case of the rice actin 1 gene and maize *Shrunken-1* introns which appear to have a role in gene expression are located in the 5' UTR. None of these introns increase gene expression if they are incorporated into constructs in an inverted orientation or are placed upstream of the promoter (Maas *et al.*, 1991; Callis *et al.*, 1987; McElroy *et al.*, 1990). Hence, their effect on gene expression is not mediated by enhancer type elements, which are independent of position and orientation (section 3.6). The maize ANT gene intron B in the 5' UTR is of a similar size and in a similar location to the intron in the 5' UTR of the *Shrunken-1* and actin 1 genes and could possibly have a similar function.

The mechanism by which these introns are able to cause an increase in gene expression is unclear. The effect of the introns discussed in this section on gene expression is dependent on their orientation and position in the gene. Hence, it is unlikely that transcriptional enhancers are

responsible for the increase in gene expression since in this case the effect would be independent of the position and orientation of the intron. Furthermore, deletions (up to 75%) of the maize alcohol dehydrogenase intron 1 do not affect enhancement or splicing (Luehrsen and Walbot, 1991; Mascarenhas *et al.*, 1990). This suggested that if enhancers were present they were close to the splice sites. Luehrsen and Walbot (1991) and Neuberger and Williams (1988) have suggested that a post-transcriptional mechanism is likely to be responsible for the enhancement of steady state mRNA levels. A conceivable mechanism which they propose is that the formation of splicing complexes in the nucleus, between the nascent mRNA and the spliceosome, may protect the RNA from nuclear RNases and lead to more mRNA being transported to the cytoplasm. If splicing is important it explains why the ability of the intron to enhance gene expression is dependent on its orientation and position, since these factors are known to affect the efficiency of splicing (Luehrsen and Walbot, 1991).

6.4.2.3 Introns in some genes contain regulatory elements which enhance gene expression

The plant introns discussed above in section 6.4.2.2 have been shown to enhance the expression of chimaeric genes when they are present within the transcription unit. It has been proposed that this regulation is mediated by a post-transcriptional mechanism rather than a transcriptional mechanism (Callis *et al.*, 1987; Luehrsen and Walbot, 1991). In vertebrate systems there are several occurrences of introns that affect the rate of transcription. Introns from rat cytochrome c gene (Evans and Scarpulla, 1988), human $\alpha 1(\text{IV})$ collagen gene (Killen *et al.*, 1988), human β -actin gene (Kawamoto *et al.*, 1988) and human immunoglobulin heavy chain gene (Gillies *et al.*, 1983) were found to contain positive regulatory sequences. *Drosophila* have both positive and negative regulatory elements in the first intron of the $\beta 3$ tubulin gene (Bruhat *et al.*, 1990). These intron specific regulatory sequences affect gene expression in an orientation and position independent manner. No similar sequences have been discovered in plants at present. However, sequences in the first intron of the soyabean actin gene (Pearson and Meagher, 1990) are similar to an enhancer sequence in the human β -actin gene (Kawamoto *et al.*, 1988), although no functional significance has been demonstrated.

6.5 FUTURE WORK

Full length cDNA clones and genomic clones containing promoter sequences are required before a thorough analysis on the control of ANT gene expression can be initiated. The following sections discuss the experiments required to continue this project towards its goal of identifying factors involved in the regulation of expression of nuclear genes encoding mitochondrial proteins.

6.5.1 Further characterisation of the *ANT* cDNA 5' UTR

The cDNA clones, pMANT1 and pMANT2, which are discussed in this thesis are not full length as determined by primer extension analysis. Full length clones are important for the progress of the project for four reasons.

- i) Full length cDNA clones will allow analysis of the 5' termini to determine whether alternative splicing or multiple initiation sites or both are responsible for the various primer extension products (section 6.4.1.3).
- ii) Subclones of the putative exon 1 can be used to screen a genomic library for a clone which contains both exon 1 and promoter sequence of the maize ANT genes. Clones containing these promoter sequences are vital to the identification of regulatory elements which are expected to control ANT gene expression.
- iii) Once the 5' UTR sequence has been cloned, a chimaeric construct containing the 5' UTR linked to a reporter gene can be used to analyse the 5' UTRs role in translational (section 6.4.1.2) or transcriptional (section 6.4.1.4) control of ANT gene expression.
- iv) The RNA sequencing experiment described in section 4.8.3 confirmed the existence of a second untranslated exon (putative exon 1) but did not identify the 5' splice site of exon 2. A full length cDNA clone will allow the postulated 5' splice site to be located (section 4.9.3).

As the majority of the *ANT* cDNA sequence is known, a cDNA library could be constructed using an *ANT* specific oligonucleotide

complimentary to a sequence close to the 5' end of the existing cDNAs. This would be advantageous for two reasons. Firstly, all the cDNA sequences synthesised would be *ANT* specific and they could be cloned directly into a plasmid at this stage. Secondly, more of the cDNAs would be full length since the *ANT* specific oligonucleotide would prime nearer the 5' terminus than an oligo dT primer. However, it is possible that secondary structure causes premature termination resulting in incomplete cDNAs (section 4.9.1).

6.5.2 Coordinated expression of ATP synthase and *ANT* transcripts

Initial northern blot analysis indicates an apparent coordination between the steady state levels of the ATP synthase β subunit and *ANT* mRNAs (section 5.5). Since a number of ATP synthase β subunit genomic clones have been isolated in this laboratory (unpublished data) its gene structure and promoter region should be analysed to search for common regulatory elements.

6.5.3 The role of the 5' UTR and its introns in gene expression

As discussed in section 6.4 the unusually long 5' UTR and its introns may have a role in transcriptional and/ or translational regulation of *ANT* gene expression. By constructing chimaeric genes which include the 5' UTRs and introns in various combinations upstream of a reporter gene their roles in gene expression can be characterised. Although there are reports of stable transgenic maize plants (Prioli and Söndahl, 1989; Shillito and Harms, 1989; Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990) a longterm drawback to these experiments is the fact that they are still not routinely transformable. A possible solution is to analyse transient expression in whole tissue. Transformation can be carried out with a particle gun as outlined by Klein *et al.* (1987, 1988). These techniques have been successful when previously used with a maize system (Oard *et al.*, 1990). Other experimental systems which could be considered include either a heterologous system, using transgenic plants such as tobacco, or a homologous system, using transient expression techniques in maize protoplasts. The homologous system is preferable when the constructs contain introns since monocot introns are often poorly spliced in a dicot system (Keith and Chua, 1986)(section 4.9). By creating specific deletions/mutations in the 5' UTRs and introns, potentially important

regulatory elements might be identified. These experiments should lead to a fuller understanding of the sequences involved in regulating *ANT* gene expression.

6.5.4 Primer extension analysis in of *ANT* genes in various maize tissues

The differential transcript analysis of *ANT* was inconclusive using the *ANT1* specific RNA probe (section 5.4.3). A different approach, which may yield better data, is quantitative primer extension analysis using an oligonucleotide which binds to both *ANT1* and *ANT2* transcripts (the ratio of primer and template are critical (Chandler and Huiet, 1991). Since the transcription initiation sites are not identical for the two genes the various extension products can be related to one of the two genes. As the primer will bind with an identical efficiency to both transcripts the different intensities of each extension product should give an indication of its abundance relative to the other. Additionally, certain extension products may be more predominant in some tissues. Quantitative primer extension would allow the study of differential expression and alternative usage of transcription initiation sites. It also should give a clearer picture of *ANT* gene expression during development.

6.5.5 Model system which may be appropriate for studying the molecular control of mitochondrial biogenesis

Ideally, to study the expression of *ANT* a 'model system' in which mitochondrial biogenesis can be modulated by external conditions is required. Due to the vital role of oxidative phosphorylation in plant metabolism a true on/off system is unlikely to exist. Two possible model systems are potentially interesting for studying mitochondrial biogenesis.

i) Etiolated seedling versus green seedling

The difference in steady state levels of transcript for both *ANT* and ATP synthase β subunit between etiolated and green tissue is significant (3.5 fold for *ANT*) (section 5.4.2). The signals which modify the transcript level and gene expression are not known. The basic characteristics of this system such as whether transcriptional control, post-transcriptional control, degradation or all of them are

responsible for the changes in transcript levels can be studied. Various growth regimes can be used to try and manipulate the *ANT* transcript steady state levels. Factors such as light, hormone treatment and substrate supply may cause differences which can be further dissected to identify the regulatory mechanisms (section 1.3.2).

ii) Roots

The biochemistry of maize and barley roots in response to salts and substrates has been comprehensively investigated (section 1.3.2). Several changes in the capacity of the cytochrome pathway in response to carbohydrate levels have been reported (Williams and Farrar, 1990; Bingham and Farrar, 1988). To date no molecular analysis of transcripts levels of OXPHOS genes has been undertaken. Further investigation of this system may reveal changes in mitochondrial and other transcript levels and would represent a model to study the regulation of expression of nuclear genes encoding mitochondrial proteins.

REFERENCES

- Ackerman J.J.H., Grove T.H. Wong G.G., Gadian D.G. and Radda G.K.
1980 Mapping of metabolites in whole animals by ^{31}P NMR using surface coils.
Nature 283, 167-170.
- Adrian G.S., McCammon M.T., Montgomery D.L. and Douglas M.G.
1986 Sequences required for delivery and localisation of the ADP/ATP translocator to the mitochondrial inner membrane.
Mol. Cell Biol. 6, 626-634.
- Allen J.F., Bennett J., Steinback K.E. and Arntzen C.J.
1981 Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems.
Nature 291, 25-29.
- ap Rees T.
1987 Compartmentation of plant metabolism.
In *The Biochemistry of Plants: A Comprehensive Treatise. Biochemistry of Metabolism*, ed D.D. Davies, 12, 87-115. New York: Academic.
- ap Rees T.
1990 Carbon metabolism in mitochondria.
In *Plant Physiology, Biochemistry and Molecular Biology* eds D.T. Dennis and D.H. Turpin, Longman Singapore Publishers, pp.106-123.
- ap Rees T., Fuller W.A. and Wright B.W.
1977 Measurements of glycolytic intermediates during the onset of thermogenesis in the spadix of *Arum maculatum*.
Biochim. Biophys. Acta. 461, 274-282.
- Aquila H., Link T.A. and Klingenberg M.
1985 The uncoupling protein from brown fat mitochondria is related to the mitochondrial ADP/ATP carrier. Analysis of sequences homologies and of folding of the protein in the membrane
EMBO J. 4, 2369-2376.
- Aquilar F., Montandon P.-E. and Stutz E.
1991 Two genes encoding the soyabean translation elongation factor eEF- α are transcribed in seedling leaves.
Plant Mol. Biol. 17, 351-360.
- Arends H. and Sebald W.
1984 Nucleotide sequence of the cloned mRNA and gene of the ADP/ATP carrier from *Neurospora crassa*.
EMBO J. 3, 377-382.

Atchison M.L.

1988 Enhancers: Mechanisms of action and cell specificity.
Ann. Rev. Cell. Biol. 4, 127-153.

Attuci S., Carde J.P., Raymond P., Saint-Gés V., Spiteri A. and Pradet A.

1991 Oxidative phosphorylation by mitochondria extracted from dry sunflower seeds.

Plant Physiol. 95, 390-398.

Azcón-Bieto J., Day D.A. and Lambers H.

1983a The regulation of respiration in the dark in wheat leaf slices.
Plant Sci. Lett. 32, 313-320.

Azcón-Bieto J., Lambers H. and Day D.A.

1983b Respiratory properties of developing bean and pea leaves.
Aust. J. Plant Physiol. 10, 237-245.

Baker A.

1985 *Nuclear genes encoding the adenine nucleotide translocator of maize mitochondria.*

Ph.D. Thesis, University of Edinburgh.

Baker A. and Leaver C.J.

1985 Isolation and sequence analysis of a cDNA encoding the ATP/ADP translocator of *Zea mays* L.
Nucleic Acids Res. 13 5857-5867.

Barkan A. and Martienssen R.A.

1991 Inactivation of maize transposon *Mu* suppresses a mutant phenotype by activating an outward-reading promoter near the end of *Mu1*.

Proc. Natl. Acad. Sci. USA 88, 3502-3506.

Barker D.G., Gallusci P., Lullien V., Khan H., Ghérardi M. and Huguet T.

1988 Identification of two groups of leghemoglobin genes in alfalfa (*Medicago sativa*) and a study of their expression during root nodule development.

Plant. Mol. Biol. 11, 761-772.

Bathgate B., Baker A. and Leaver C.J.

1989 Two genes encode the adenine nucleotide translocator of maize mitochondria: Isolation, characterisation and expression of the structural genes.

Eur. J. Biochem. 183, 303-310.

Beckett A., Hawker L.E., Sleigh M.A., Madelin M.F. and Round F.E.
1971 Eukaryotes.

In *Micro-organisms: Function, Form, and Environment*. ed Hawker L.E.
and Linton A.H. Edward Arnold Ltd.

Beevers H.

1980 The role of the glyoxylate cycle.

In *The Biochemistry of Plants*, Vol 4, ed P.K. Stumpf, Academic Press,
New York, pp 117-129.

Benfey P.N. and Chua N.-H.

1990 The Cauliflower Mosaic Virus 35S promoter: Combinatorial
regulation of transcription in plants.

Science 250, 959-966.

Benkel B.F. and Hickey D.A.

1987 A *Drosophila* gene is subject to glucose repression.

Proc. Natl. Acad. Sci. USA 84, 1337-1339.

Bewley J.D. and Black M.

1985 *Seeds: physiology of development and germination*.

Plenum Press, New York.

Bingham I.J. and Farrar J.F.

1988 Regulation of respiration in roots of barley.

Physiologia Pl. 73 278-285.

Bingham I.J. and Farrar J.F.

1989 Activity and capacity of respiratory pathways in barley roots
deprived of inorganic nutrients.

Plant Physiol. Biochem. 27, 847-854.

Birnboim H.C. and Doly J.

1979 A rapid alkaline extraction procedure for screening
recombinant plasmid DNA.

Nucleic Acids Res. 7, 1513-1523

Biswas T.K.

1990 Control of mitochondrial gene expression in the yeast
Saccharomyces cerevisiae.

Proc. Natl. Acad. Sci. USA 87, 9338-9342.

Bolivar F. and Backman K.

1979 Plasmids of *Escherichia coli* as cloning vectors.

Methods Enz. 68, 245.

- Borck K., Beggs J.D., Brammer W.J., Hopkins A.S. and Murray N.E.
1976 The construction in vitro of transducing derivatives of phage lambda.
Mol. Gen. Genet. 146, 199.
- Bown and Nicholls
1985 An investigation into the role of photosynthesis in regulating ATP levels and rates of H⁺ efflux in isolated mesophyll cells.
Plant Physiol. 79, 928-934.
- Boyer H.W. and Roulland-Dussoix D.
1969 A complementation analysis of the restriction and modification of DNA in *Escherichia coli*.
J. Mol. Biol. 41, 459.
- Brand A.H., Breeden L., Abraham J., Sternglanz R. and Nasmyth K.
1985 Characterisation of a "silencer" in yeast: A DNA sequence with properties opposite to those of a transcriptional enhancer.
Cell 41, 41-48.
- Bremer K.
1985 Summary of green plant phylogeny and classification.
Cladistics 1, 369-385.
- Brouquisse R., James F., Raymond P. and Pradet A.
1991 Study of glucose starvation in excised maize root tips.
Plant Physiol. 96, 619-626.
- Brown J.W.S.
1986 A catalogue of splice junction and putative branch point sequences from plant introns.
Nucleic Acids Res. 14, 9549-9559.
- Bruhat A., Tourmeute S., Chapel S., Sobrier M.L., Couderc J.L. and Dastugue B.
1990 Regulatory elements in the first intron contribute to transcriptional regulation of the β_3 tubulin gene by 20-hydroxyecdysone in *Drosophila* kc cells.
Nucleic Acids Res. 18, 2861-2867.
- Bryce J.H. and ap Rees T.
1985 Rapid decarboxylation of the products of dark fixation of CO₂ in roots of *Pisum* and *Plantago*.
Phytochemistry 24, 1635-1638.
- Callis J., Fromm M. and Walbot V.
1987 Introns increase gene expression in cultured maize cells.
Genes and Dev. 1, 1183-1200.

Campbell S.M. and Rosen J.M.

1984 Comparison of the whey acidic protein genes of the rat and mouse.

Nucleic Acids Res. 12, 8685-

Cech T.R.

1986 The generality of self splicing RNA: Relationship to nuclear mRNA splicing.

Cell 44, 207-210.

Celenza D.L. and Carlson M.

1986 A yeast gene that is essential for release from glucose repression encodes a protein kinase.

Science 233, 1175-1180.

Chance B. and Williams G.R.

1955 Respiratory enzymes in oxidative phosphorylation. I Kinetics of oxygen utilisation.

J. Biol. Chem. 217, 385-393.

Chandler P.M. and Huiet L.

1991 Primer extension studies on α -amylase mRNAs in barley aleurone. I Characterisation and quantification of the transcripts.

Plant Mol. Biol. 16, 627-636.

Cheng C-L., Acedo G.N. Cristinsin M. and Conkling M.A.

1992 Sucrose mimics the light induction of *Arabidopsis* reductase gene transcription.

Proc. Natl. Acad. Sci. USA 89, 1861-1864.

Chomyn A. and Lai S.S.A.T.

1990 Regulation of expression of nuclear and mitochondrial genes for mammalian NADH dehydrogenase.

In *Structure, function and biogenesis of energy transfer systems*. ed. E. Quagliariello, S. Papa, F. Palmieri and C. Saccone, Elsevier Science Publications, pp 179-185.

Claussen W., Loveys B.R. and Hawker J.S.

1986 Influence of sucrose and hormones on the activity of sucrose synthase and invertase in detached leaves and leaf sections of egg plants (*Solanum melangona*).

J. Plant Physiol. 124, 345-357.

Cocucci M.C. and Marré E.

1973 The effect of cycloheximide on respiration, protein synthesis and adenosine nucleotide levels in *Rhodotorula gracilis*.

Plant Sci. Lett. 1, 293-301.

Costanzo M.C. and Fox T.D.

1990 Control of mitochondrial gene expression in *Saccharomyces cerevisiae*.

Ann. Rev. Genet. 24, 91-113.

Couée I., Defontaine S., Carde J-P. and Pradet A.

1992 Effects of anoxia on mitochondrial biogenesis in rice shoots. Modification of an *in Organello* translation characteristics.

Plant Physiol. 98, 411-421.

Cozens A.L., Runswick M.J. and Walker J.E.

1989 DNA sequences of two expressed nuclear genes for human mitochondrial ADP/ATP translocase.

J. Mol. Biol. 206, 261-280.

Cross R.L.

1981 The mechanism and regulation of ATP synthesis by F1 ATPases.

Ann. Rev. Biochem. 50, 681-714.

Crouse G.F., Leys E.J. McEwan R.N., Frayne E.G. and Kellems R.E.

1985 Analysis of the mouse *dhfr* promoter region: Existence of a divergently transcribed gene.

Mol. Cell Biol. 5, 1847

Davies D.D.

1979 The central role of phosphoenol pyruvate in plant metabolism.

Ann. Rev. Plant Physiol. 30, 131-158.

Day D.A. and Lambers H.

1983 The regulation of glycolysis and electron transport in roots.

Physiologia Pl. 58, 155-160.

Day D.A. and Wiskich J.T.

1984 Transport processes of isolated mitochondria.

Physiol. Vég. 22, 241-261.

Day D.A., De Vos O.C., Wilson D. and Lambers H.

1985 The regulation of respiration in the leaves and roots of two *Lolium perenne* populations with contrasting mature leaf respiration rates and yield.

Plant Physiol. 78, 678-683.

De Klerk-Kiebert Y.M., Kneppers T.J.A., Plas van der L.H.W.

1982 Influence of chloramphenicol on growth and respiration of soyabean (*Glycine max L.*) suspension cultures.

Physiologia Pl. 55, 98-102.

De Visser R., Spreen Brouwer K. and Posthumus F.

1986 Alternative path mediated ATP synthesis in roots of *Pisum sativum* upon nitrogen supply.
Plant Physiol. 80, 295-300.

Dean C., Tamaki S., Dunsmuir P., Favreau M., Katayama C., Dooner H. and Bedbrook J.

1986 mRNA transcripts of several plant genes are polyadenylated at multiple sites *in vivo*.
Nucleic Acids Res. 14, 2229-2240.

Deák F., Barta E., Mestric S., Biesold M. and Kiss I.

1991 Complex pattern of alternative splicing generates unusual diversity in the leader sequence of the chicken link protein mRNA.
Nucleic Acids Res. 19, 4983-4990.

Deikman J. and Fisher R.L.

1985 Interaction of a DNA binding factor with the 5'-flanking region of an ethylene-responsive fruit ripening gene from tomato.
EMBO J. 7, 3315-3320.

Devereux J., Haerberli P. and Smithies O.

1984 A comprehensive set of sequence analysis programmes for the VAX.
Nucleic Acids Res. 12, 387-395.

Dewey R.E., Schuster A.M., Levings III C.S. and Timothy D.H.

1985 Nucleotide sequence of F₀-ATPase proteolipid (subunit 9) gene of maize mitochondria.
Proc. Natl. Acad. Sci. USA 82, 1015-1019.

Dignam J.D., Lebovitz R.M. and Roeder R.G.

1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei.
Nucleic Acids Res. 11, 1475-1489.

Ditta G., Stanfield S., Corbin D. and Helinski D.R.

1980 Broad host range cloning system for gram negative bacteria - Construction of a gene bank of *Rhizobium melilotis*.
Proc. Natl. Acad. Sci. USA 77, 7347-7351.

Dizengremel P.

1985 Potato respiration: electron transport pathways.
In *Potato Physiology*. ed P.H. Li, Academic Press, Orlando, pp. 354-388.

- Douce R
 1985 *Mitochondria in Higher Plants. Structure, Function and Biogenesis.*
 Academic Press, Orlando.
- Douce R. and Neuburger M.
 1989 The uniqueness of plant mitochondria.
Ann. Rev. Plant Physiol. Plant Mol. Biol. 40, 371-414.
- Douce R., Bligny R., Brown D., Dorne A-J., Genix P. and Roby C.
 1991 Autophagy triggered by sucrose deprivation in sycamore (*Acer pseudoplatanus*) cells.
 In *Compartmentation of Plant Metabolism in Non-Photosynthetic Tissues*,
 SEB Seminar Series Vol 42, ed M.J. Emes, Cambridge University Press, pp
 127-145.
- Douce R., Brouquisse R. and Journet E.-P.
 1987 Electron transfer and oxidative phosphorylation in plant
 mitochondria.
 In *The Biochemistry of Plants: A Comprehensive Treatise. Biochemistry
 of Metabolism*, ed D.D. Davies, 11, 177-211. New York: Academic.
- Downie J.A. and Garland P.B.
 1973 An antimycin A and cyanide-resistant variant of *Candida utilis*
 arising during copper limited growth.
Biochem J. 134, 1051-1061.
- Draper J., Scott R., Armitage P. and Walden R.
 1988 *Plant genetic transformation and gene expression: A laboratory
 manual.*
 Blackwell Scientific Publications.
- Dry I.B. and Wiskich J.T.
 1982 Role of the external adenosine triphosphate/adenosine
 diphosphate ratio in the control of plant mitochondrial respiration.
Arch. Biochem. Biophys. 217, 72-79.
- Dry I.B., Bryce J.H. and Wiskich J.T.
 1987 Regulation of mitochondrial respiration.
 In *The Biochemistry of Plants: A Comprehensive Treatise. Biochemistry
 of Metabolism*, ed D.D. Davies, 11, 213-252. New York: Academic.
- Duke S.H. and Duke S.O.
 1984 Light control of extractable nitrate reductase activity in higher
 plants.
Physiologia Pl. 62, 485-493.

- Dynan W.S.
1986 Promoters for housekeeping genes.
TIGS 2, 196-197.
- Ehrenshaft M. and Brambl R.
1990 Respiration and mitochondrial biogenesis in germinating embryos of maize.
Plant Physiol. 93, 295-304.
- Elthon E.T., Nickels R.L. and McIntosh L.
1987 Mitochondrial events during development of thermogenesis in *Sauromatum guttatum* (Schott).
Planta 180, 82-89.
- Evans M.J. and Scarpulla R.C.
1988 Both upstream and intron elements are required for elevated expression of the rat somatic cytochrome c gene in COS-1 cells.
Mol. Cell Biol. 8, 35-41.
- Evans M.J. and Scarpulla R.C.
1989 Interaction of nuclear factors with multiple sites in the somatic cytochrome c promoter. Characterisation of upstream NRF-1, ATF, and intron Sp1 recognition sequences.
J. Biol. Chem. 264, 14361-14368.
- Fang R.X., Nagy F., Sivasubramaniam S. and Chua N.-H.
1989 Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants.
Plant Cell 1, 141-150.
- Farell L.E., Trawick J.D. and Poyton R.O.
1990 Mitochondrial-nuclear interactions: Transcription of nuclear COX genes in yeast is reduced in cells that lack a mitochondrial genome. In *Structure, function and biogenesis of energy transfer systems*. ed. E. Quagliariello, S. Papa, F. Palmieri and C. Saccone, Elsevier Science Publications, pp 131-134.
- Farrar J.F.
1981 Respiration of barley roots: Its relation to growth, substrate supply and the illumination of the shoot.
Ann. Bot. 48, 53-63.
- Farrar J.F. and Williams J.H.H.
1991 Control of the rate of respiration in roots: compartmentation, demand and the supply of substrate. In *Compartmentation of Plant Metabolism in Non-Photosynthetic Tissues*, SEB Seminar Series Vol 42, ed M.J. Emes, Cambridge University Press, pp. 167-188.

Feinberg A.P. and Vogelstein B.

1984 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity.

Addendum Analyt. Biochem. 137, 266-267.

Forsburg S.L. and Guarente L.

1989 Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisiae*.

Ann. Rev. Cell Biol. 5, 153-180.

Fox T.D.

1986 Nuclear gene products required for translation of specific mitochondrially coded mRNAs in yeast.

TIGS 97-100.

Fromm M.E., Morrish F., Armstrong C. and Williams R., Thomas J. and Klein T.M.

1990 Inheritance and expression of chimaeric genes in the progeny of transgenic maize plants.

Biotechnology 8, 833-839.

Gardestöm P. and Edwards G.E.

1985 Leaf mitochondria (C3 + C4 + CAM).

In *Higher Plant Cell Respiration, Encyclopedia of Plant Physiology, New Series Vol 18*, eds R. Douce and D.A. Day, Springer-Verlag, Berlin, pp. 314-346.

Geidusschek E.P. and Tocchini-Valentini G.P.

1988 Transcription by polymerase III.

Ann. Rev. Biochem. 57, 873-914.

Gierl A., Saedler H. and Peterson P.A.

1989 Maize transposable elements.

Ann. Rev. Genet. 23, 71-85.

Gil G., Brown M.S. and Goldstein J.L.

1986 Cytoplasmic 3-hydroxy-3-methylglutaryl Coenzyme A synthase from hamster. II Isolation of the gene and characterisation of the 5' flanking region.

J. Biol. Chem. 261, 3717-3724.

Gilbert W.

1978 Why genes in pieces.

Nature 271, 501.

Gilbert W.

1985 Genes in pieces revisited.

Science 228, 823-824,

Gillies S.D., Morrison S.L., Oi V.T. and Tonegawa S.

1983 A tissue specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene.
Cell 33, 717-728.

Goodall G.J. and Filiowicz W.

1989 The AU-rich sequences present in the introns of plant nuclear mRNAs are required for splicing.
Cell 58, 473-483.

Goodall G.J. and Filiowicz W.

1991 Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants.
EMBO J. 10, 2635-2644.

Gordon-Kamm W.J., Spencer T.M., Mangano M.L., Adams T.R. Daines R.J., Start W.G., O'Brien J.V., Chambers S.A., Adams Jr W.R., Willets N.G., Rice T.B., Mackey C.J., Krueger R.W., Kausch A.P. and Lemaux P.G.

1990 Transformation of maize cells and regeneration of fertile transgenic plants.
Plant Cell 2, 503-618.

Göller M., Hampp R. and Ziegler H.

1982 Regulation of the cytosolic adenylate ratio as determined by rapid fractionation of mesophyll protoplasts of oat. Effect of electron transfer inhibitors and uncouplers.
Planta 156, 255-263.

Graham D.

1980 Effects of light on 'dark' respiration.
In *The Biochemistry of Plants: A Comprehensive Treatise. Metabolism and Respiration*, ed D.D. Davies, 2, 525-579. New York, Academic.

Graham I.G., Leaver C.J. and Smith S.M.

1992 Induction of malate synthase gene expression in senescent and detached organs of cucumber.
Plant Cell 4, 349-357.

Graham R.W., Van Doren K., Bektesh S. and Candido E.P.M.

1988 Maturation of the major ubiquitin gene transcript in *Caenorhabditis elegans* involves the acquisition of a trans-spliced leader.
J. Biol. Chem. 263, 10415-10419.

Gribskov M. and Burgess R.R.

1986 Sigma factors from *E. coli*, *B. subtilis*, phage SPO1, and phage T4 are homologous preitiens.
Nucleic Acids Res. 14, 6745-6763.

Groen A.K., Wanders R.J.A., Westerhoff H.V. van der Meer R. and Tager J.M.

1982 Quantification of the contribution of various steps to the control of mitochondrial respiration.
J. Biol. Chem. 257, 2754-2757.

Gruissem W.

1989 Chloroplast gene expression: How plants turn their plastids on.
Cell 56, 161-170.

Guarente L and Mason T.

1983 Heme regulates transcription of the CYC1 gene in *Saccharomyces cerevisiae* via an upstream activation site.
Cell 32, 1279-1286.

Guarente L., Lalonde B., Gifford P. and Alani E.

1984 Distinctly regulated tandem upstream activation sites mediate catabolite expression of the CYC1 gene of *Saccharomyces cerevisiae*.
Cell 36, 503-511.

Hanley B.A. and Schuler M.A.

1988 Plant intron sequences: evidence for distinct groups of introns.
Nucleic Acids Res. 14, 7159-7176.

Hashimoto H., Uchimiya H. and Kato A.

1992 Unpublished but can be accessed from the EMBL, Genbank sequence database with the accession number D12637.

Hawkins J.D.

1988 A survey of introns and exon lengths.
Nucleic Acids Res. 16, 9893-9908.

Heber U.

1974 Metabolite exchange between chloroplasts and cytoplasm.
Ann. Rev. Plant Physiol. 25, 393-421.

Heldt H.W., Klingenberg M. and Milovancev M.

1972 Differences between the ADP/ATP ratios in the mitochondrial matrix and the extra-mitochondrial space.
Eur. J. Biochem. 30, 434-440.

Hemberg T.

1985 Potato rest.
In *Potato Physiology*. ed P.H. Li, Academic Press, Orlando, pp. 354-388.

Hennighausen L. and Luban H.

1985 Interaction of protein with DNA invitro.
Methods Enz. 152, 721-755.

Hilgarth C., Sauer N. and Tanner W.

1991 Glucose increases the expression of the ATP/ADP translocator and glyceraldehyde-3-phosphate dehydrogenase genes in *Chlorella*.
J. Biol. Chem. 266, 24044-24047.

Hill S.

1990 *The regulation of mitochondrial function during early seedling development in cucumber (Cucumis sativus L.)*.
Ph.D. Thesis. University of Edinburgh.

Hiser C. and McIntosh L.

1990 Alternative oxidase of potato is an integral membrane protein synthesised de novo during aging of tuber slices.
Plant Physiol. 93, 312-318.

Holian A., Owen C.S. and Wilson D.F.

1977 Control of respiration in isolated mitochondria: Quantitative evaluation of the dependence of the respiratory rates on [ATP], [ADP] and [Pi].
Arch. Biochem. Biophys. 181, 164-171.

Houldsworth J. and Attardi G.

1988 Two distinct genes for ADP/ATP translocase are expressed at the mRNA level in adult human liver.
Proc. Natl. Acad. Sci. USA 85, 377-381.

Huang J., Hack E., Thornburg R.W. and Myers A.M.

1990 A yeast mitochondrial leader peptide functions in vivo as a dual targeting signal for both chloroplasts and mitochondria.
Plant Cell 2, 1249-1260.

Hussey P.J., Haas N., Hunsperger J., Larkin J., Sunstad D.P. and Silflow C.D.

1990 The β -tubulin gene family in *Zea mays*: two differentially expressed β -tubulin genes.
Plant Mol. Biol. 15, 957-972.

Huynh T.V., Young R.A. and Davis R.W.

1985 Constructing and screening cDNA libraries in λ gt10 and λ gt11.
In *DNA cloning: A practical approach* (ed. Glover D.M.), vol. 1, p. 49 IRL Press, Oxford.

Hylton C.M., Rawsthorne S., Smith A.M., Jones D.A. and Woolhouse H.W.

1988 Glycine decarboxylase is confined to the bundle sheath cells of leaves of C3-C4 intermediate species.

Planta 175, 52-459.

Inoue Y., Kobay-Ashi Y., Shibata K. and Heber U.

1978 Synthesis and hydrolysis of ATP by intact chloroplasts under flash illumination and in darkness.

Biochim. Biophys. Acta 504, 142-152.

Isaac P.G., Brennicke A., Dunbar S.M. and Leaver C.J.

1985 The mitochondrial genome of fertile maize (*Zea mays* L.) contains two copies of the gene encoding the α subunit of F1-ATPase.

Curr. Genet. 10, 321-328.

Itakura K., Rossi J.J. and Wallace R.B.

1984 Synthesis and use of synthetic oligonucleotides.

Annu. Rev. Biochem. 53, 323-356.

Jackson I.J.

1991 A reappraisal of non- consensus mRNA splice sites.

Nucleic Acids Res. 19, 3795-3798.

Jacobus W.E., Moreadilla R.W. and Vandegaer K.M.

1982 Mitochondrial respiratory control. Evidence against the regulation of respiration by extramitochondrial phosphorylation potentials or by [ATP]/[ADP] ratios.

J. Biol. Chem. 257, 2397-2402.

Jefferson R.A.

1987 Assaying chimeric genes in plants: The GUS gene fusion system.

Plant Mol. Biol. Report. 5, 387-405.

Jobling S.A. and Gehrke L.

1987 Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence.

Nature 325, 622-625.

Jolivet Y., Pireaux J-C. and Dizengremel P.

1990 Changes in properties of barley leaf mitochondria isolate from the NaCl treated plants.

Plant Physiol. 94, 641-646.

Joshi C.P.

1987a An inspection of the domain between putative TATA box and translation start site in 79 plant genes.
Nucleic Acids Res. 15, 6643-6653.

Joshi C.P.

1987b Putative polyadenylation signals in nuclear genes of higher plants: a compilation and analysis.
Nucleic Acids Res. 15 9627-9640.

Journet E.P., Bligny R. and Douce R.

1986 Biochemical changes during sucrose deprivation in higher plant cells.
J. Biol. Chem. 261, 3193-3199.

Kadonaga J.T., Jones K.A. and Tjian R.

1986 Promoter specific activation of RNA polymerase II transcription by Sp1.
TIBS 11, 20-23.

Katz L., Kingsbury D.T. and Helinski D.R.

1973 Stimulation by cyclic adenosine monophosphate of plasmid deoxyribonucleic acid replication and catabolite repression of the plasmid deoxyribonucleic acid and protein relaxation complex.
J. Bacteriol. 114, 577-591.

Kaufman P.B., Ghosheh N.S., LaCroix J.D., Sarvjit L.S. and Ikuma H.

1973 Regulation of invertase levels in *Avena* stem segments of gibberellic acid, sucrose, glucose and fructose.
Plant Physiol. 52, 221-228.

Kawamoto T, Makino K., Niwa H., Sugiyama H., Kimura S., Amemura M., Nakata A.,

Kakunaga T.

1988 Identification of the human *b*-actin enhancer and its binding factor.
Mol. Cell Biol. 8, 267-272.

Käppeli O.

1986 Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts.
Ad. Microb. Physiol. 28, 189-209.

Keith B. and Chua N.-H.

1986 Monocot and dicot pre-mRNAs are processed with different efficiencies in transgenic tobacco.
EMBO J. 5, 2419-2425.

Kagawa Y. and Ohta, S.

1990 Regulation of mitochondrial ATP synthesis in mammalian cells by transcriptional control.
Int. J. Biochem. 22, 219-

Kidd F. and West C.A.

1925

Rep. Food Invest. Board, 1924 pp. 27-32.

Killen P.D., Burbelo P.D., Martin G.R. and Yamada Y.

1988 Characterisation of the promoter for the $\alpha 1$ (IV) collagen gene.

DNA sequences within the first intron enhance transcription.

J. Biol. Chem. 263, 12310-12314.

King M.P. and Attardi G.

1988 Injection of mitochondria into the human cells leads to a rapid replacement of the endogenous mitochondrial DNA.

Cell 52, 811-819.

Klein T.M., Fromm M.E., Gradziel T. and Sanford J.C.

1988 Factors influencing gene delivery into *Zea mays* cells by high velocity microprojectiles.

Biotechnology 6, 923-926.

Klein T.M., Wolf E.D., Wu R. and Sanford J.C.

1987 High-velocity microprojectiles for delivering nucleic acids into living cells.

Nature 327, 70-73.

Klingenberg M.

1961

Biochem. Z. 335, 263-272.

Klingenberg M.

1976

In *The Enzymes of Biological Membranes. Vol. 3: Membrane Transport*, pp. 383-438, Plenum Publishing Corp., New York.

Klingenberg M.

1980 The ADP/ATP translocator in mitochondria, a membrane potential controlled transport.

J. Membr. Biol. 56, 97-105.

Klingenberg M.

1988 Molecular aspects of the nucleotide carrier.

Arch. Biochem. Biophys. 270, 1-14.

Klingenberg M.

1990 Mechanism and evolution of the uncoupling protein of brown adipose tissue.

TIBS 15, 108-112.

- Koch K.E., Nolte K.D., Duke E.R., McCarty D.R. and Avigne W.T.
1992 Sugar levels modulate differential expression of maize sucrose synthase genes.
Plant Cell 4, 59-69.
- Kolarov J., Kolarova N. and Nelson N.
1990 A third ADP/ATP translocator gene in yeast.
J. Biol. Chem. 265, 12711-12716.
- Koller B., Fromm H., Galun E. and Edelman M.
1987 Evidence for *In vivo* trans splicing of pre-mRNAs in tobacco chloroplasts.
Cell 48, 111-119.
- Kozak L.P., Britton J.H., Kozak U.C. and Wells J.M.
1988 The mitochondrial uncoupling protein gene correlation of exon structure to transmembrane domains.
J. Biol. Chem. 263, 12274-12277.
- Kozak M.
1989 Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs.
Mol. Cell Biol. 9, 5134-5142.
- Krieg P.A.
1990 Improved synthesis of full-length RNA probe at reduced incubation temperatures.
Nucleic Acids Res. 18, 6463.
- Kromer S., Stitt M. and Heldt H.W.
1988 Mitochondrial oxidative phosphorylation participating in photosynthetic metabolism of a leaf cell.
FEBS Letters. 226, 352-356.
- Ku D.-H., Kagan J., Chen S.-T., Chang C.-D., Baserga R. and Wurzel J.
1990 The human fibroblast adenine nucleotide translocator gene. Molecular cloning and sequence.
J. Biol. Chem. 265, 16060-16063.
- Kuroiwa T., Fujie M. and Kuroiwa H.
1992 Studies on the behaviour of mitochondrial DNA: Synthesis of mitochondrial DNA occurs actively in a specific region above the quiescent center in the root meristem of *Pelargonium zonale*.
J. Cell Sci. 101, 483-493.

Küster U., Bohneusack R. and Kunz W.

1976 Control of oxidative phosphorylation by the extra mitochondrial ATP/ADP ratio.

Biochim. Biophys. Acta 440, 391-402.

Lambers H

1985 Respiration in intact plants and tissues: Its regulation and dependance on environmental factors, metabolism and invaded organisms.

In *Higher Plant Cell Respiration, Encyclopedia of Plant Physiology, New Series Vol 18*, eds R. Douce and D.A. Day, Springer-Verlag, Berlin, pp. 418-473.

Lambers H.

1990 Oxidation of mitochondrial NADH and the synthesis of ATP.

In *Plant Physiology, Biochemistry and Molecular Biology* eds D.T. Dennis and D.H. Turpin, Longman Singapore Publishers, pp.124-143.

Lambers H., Posthumus F., Stulen I., Lanting L., van de Dijk S.J. and Hofstra R. 1981

Energy metabolism of *Plantago major* ssp. *major* as dependent on the supply of mineral nutrients.

Physiologia Pl. 51, 245-252.

Lambowitz A.M., Sabourin J.R., Bertrand H., Nickels R. and McIntosh L.

1988 Immunological identification of the alternative oxidase of *Neurospora crassa* mitochondria.

Mol Cell Biol 9, 1362-1364.

Lance C. and Rustin P.

1984 The central role of malate in plant metabolism.

Physiol. Vég. 22, 625-641.

Lance C., Chauveau M. and Dizengremel P.

1985 The cyanide-resistant pathway of plant mitochondria.

In *Higher Plant Cell Respiration, Encyclopedia of Plant Physiology, New Series Vol 18*, eds R. Douce and D.A. Day, Springer-Verlag, Berlin, pp. 202-247.

Langridge P. and Feix G.

1983 A zein gene of maize is transcribed from two widely separated promoter regions.

Cell 34, 1015-1022.

Laskey R.A. and Mills A.D.

1975 Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography.

Eur. J. Biochem. 56 335-341.

Laties G.G.

1982 The cyanide-resistant, alternative respiration path in higher plant respiration.

Ann. Rev. Plant Physiol. 33, 519-535.

Lawson J.E. and Douglas M.G.

1988 Seperate genes encode functionally equivalent ADP/ATP carrier proteins in *Saccharomyces cerevisiae*.

J. Biol. Chem. 263, 14812-14818.

Laz T.M., Pietras D.F. and Sherman F.

1984 Differential regulation of the duplicated isocytochrome c genes in yeast.

Proc. Natl. Acad. Sci. USA 81, 4475-479.

Lee J.S.-L. and Warmke H.E.

1979 Organelle size and number in fertile and T-cytoplasmic male-sterile corn.

Am. J. Bot. 66, 141-148.

Leibold E.A. and Munro H.N.

1988 Cytoplasmic protein binds into a highly conserved sequence in the 5' untranslated region of ferritin heavy and light-subunit mRNAs.

Proc. Natl. Acad. Sci. USA 85, 2171-2175.

Li K., Hodge J.A. and Wallace D.C.

1990 OXBOX a positive transcriptional element of the heart skeletal muscle ADP/ATP translocator gene.

J. Biol. Chem. 265, 20585-20588.

Li K., Warner C.K., Hodge J.A., Minoshima S., Kudoh J., Fukuyama R., Maekawa M., Shimizu Y., Shimizu N. and Wallace D.C.

1989 A human muscle adenine nucleotide translocator gene has four exons, islocated on chromosome 4, and is diferentially expressed.

J. Biol. Chem. 264, 13998-14004.

Lin Y.-S. and Green M.R.

1988 Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1a- and cyclic AMP-inducible promoters.

Proc. Natl. Acad. Sci. USA 85, 3396-3400.

Logemann J., Schell J. and Willmitzer L.

1987 Improved method for the isolation of RNA from plant tissues.

Analytical Biochemistry 163, 16-20.

Luehrsen K.R. and Walbot V.

1991 Intron enhancement of gene expression and the splicing efficiency of introns in maize cells.

Mol. Gen. Genet. 225, 81-93.

Lunardi J. and Attardi G.

1991 Differential regulation of expression of the multiple ADP/ATP translocase genes in human cells.

J. Biol. Chem. 266, 16534-16540.

Luo X., Park K., Lopez-Cassillas F. and Kim K.-H.

1989 Structural features of the acetyl-CoA carboxylase gene: Mechanisms for the generation of mRNAs with 5'-end heterogeneity.

Proc. Natl. Acad. Sci. USA 86, 4042-4046.

Maas C., Laufs J., Grant S., Korfhage C. and Werr W.

1991 The combination of a novel stimulatory element in the first exon of the maize *Shrunken-1* gene with the following intron 1 enhances reporter gene expression up to 1000 fold.

Plant Mol. Biol. 16, 199-207.

Maas C., Schaal S. and Werr W.

1990 A feedback control element near the transcription start site of the maize *Shrunken* gene determines promoter activity.

EMBO J. 9, 3447-3452.

MacDougall A.J. and ap Rees T.

1991 Control of the Krebs cycle in *Arum spadix*.

J. Plant Physiol. 137, 683-690.

Marykwas D.L. and Fox T.D.

1989 Control of the *Saccharomyces cerevisiae* regulatory gene *PET494*: Transcriptional repression by glucose and translational induction by oxygen.

Mol. Cell Biol. 9, 484-491.

Mascarenhas D., Mettler I.J., Pierce D.A. and Lowe H.W.

1990 Intron-mediated enhancement of heterologous gene expression in maize.

Plant Mol. Biol. 15, 913-920.

Masuda H., Takahashi T. and Sugawara S.

1988 Acid and alkaline invertases in suspension cultures of sugar beet cells.

Plant Physiol. 86, 312-317.

McCashin B.G., Cossins E.A. and Cavin D.T.

1988 Dark respiration during photosynthesis in wheat leaf slices.
Plant Physiol. 87, 155-161.

McElroy D., Zhang W., Cao J. and Wu R.

1990 Isolation of an efficient actin promoter for use in rice transformation.
Plant Cell 2, 163-171.

McGlasson W.B., Palmer J.K., Vendrell M. and Brady C.J.

1971
Aust. J. Biol. Sci. 24, 1103-1114.

Meeuse B.J.D.

1975 Thermogenic respiration in Araoids.
Ann. Rev. Plant Physiol. 26, 117-126.

Messing J.

1979 A multipurpose cloning system based on single-stranded DNA bacteriophage M13.
Recomb. DNA Tech. Bull. 2, 43.

Michel F. and Dujon B.

1983 Conservation of RNA secondary structure in two intron families including mitochondrial, chloroplast, and nuclear encoded members.
EMBO J. 2, 33-38.

Mitchell P.

1980
Ann. N.Y. Acad. Sci. 341, 564-584.

Mitchell P.J. and Tjian R.

1989 Transcriptional regulation in mammalian cells by sequence specific DNA binding proteins.
Science 245, 371-378.

Moore A.L. and Rich P.R.

1980 The bioenergetics of plant mitochondria.
TIBS 5, 284-288.

Moore A.L. and Rich P.R.

1985 Organization of the respiratory chain and oxidative phosphorylation.
In *Higher Plant Cell Respiration, Encyclopedia of Plant Physiology*, New Series Vol 18, eds R. Douce and D.A. Day, Springer-Verlag, Berlin, pp. 134-172.

Moreno-Sánchez R.

1985 Contribution of the translocator of adenine nucleotides and the ATP synthase to the control of oxidative phosphorylation and arsenylation in liver mitochondria.

J. Biol. Chem. 260, 12554-12560.

Morohashi Y.

1980 Development of mitochondrial activity in pea cotyledons following imbibition; Influence of the embryonic axis.

J. Exp. Bot. 31, 805-812.

Morohashi Y., Bewley J.D. and Yeung E.C.

1981 Biogenesis of mitochondria in imbibed peanut cotyledons: Influence of the axis.

J. Exp. Bot. 32, 605-613.

Mulligan R.M., Leon P. and Walbot V.

1991 Transcriptional and post-transcriptional regulation of maize mitochondrial gene expression.

Mol. Cell Biol. 11, 533-543.

Murray N.E., Brammer W.J. and Murray K.

1977 Lambdoid phages that simplify the recovery of in vitro recombinants.

Mol. Gen. Genet. 150, 53-61.

Møller I.M. and Lin W.

1986 Membrane bound NAD(P)H dehydrogenases in higher plant cells.

Ann. Rev. Plant Physiol. 37, 309-334.

Møller I.M. and Palmer J.M.

1981 Properties of the oxidation of exogenous NADH by plant mitochondria. Evidence against a phosphatase or a nicotinamide nucleotide transhydrogenase being responsible for NADPH oxidation.

Biochim. Biophys. Acta 638, 225-233.

Møller I.M. and Palmer J.M.

1982 Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner surface of the inner membrane of plant mitochondria.

Physiologia Pl. 54, 267-274.

Nebiolo and White,

1985 Corn mitochondrial protein synthesis in response to heat shock.

Plant Physiol. 79, 1129-1132.

- Neckelman N., Li K., Wade R.P., Shuster R. and Wallace D.C.
1987 cDNA sequence of a human skeletal muscle ADP/ATP translocator: Lack of a leader peptide, divergence from a fibroblast translocator cDNA, and coevolution with mitochondrial DNA genes.
Proc. Natl. Acad. Sci. USA 84, 7580-7584.
- Neuberger M.S. and Williams G.T.
1988 The intron requirement for immunoglobulin gene expression is dependant upon the promoter.
Nucleic Acids Res. 16, 6713-6724.
- Newton K.J. and Walbot V.
1985 Maize mitochondria synthesize organ specific polypeptides.
Proc. Natl. Acad. Sci. USA 82, 6879-6883.
- Nicholls D.G.
1984
Physiol. Rev. 64, 1-64.
- Noodén L.D.
1988 The phenomena of senescence and aging.
In *Senescence and aging in plants*. eds L.D. Noodén and A.C. Leopold, Academic Press, New York, pp 1-50.
- Oard J.H., Paige D.F., Simmonds J.A. and Gradziel T.M.
1990 Transient gene expression in maize, rice, and wheat cells using an airgun apparatus.
Plant Physiol. 92, 334-339.
- Ogren W.L.
1984 Photorespiration: pathways, regulation and modification.
Ann. Rev. Plant Physiol. 35, 415-442.
- Olesen J.T. and Guarente L.
1990 The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: Model for the HAP2/3/4 complex.
Genes and Dev. 4 1714-1729.
- Olesen J.T., Hahn S. and Guarente L.
1987 Yeast HAP2 and HAP3 activators both bind to the CYC1 upstream activation site UAS2 in an interdependant manner.
Cell 51, 953-961.
- Owen J.H., Hetherington, A.M. and Wellburn A.R.
1987a Inhibition of respiration in protoplasts from meristematic tissues by abscisic acid in the presence of calcium ions.
J. Exp. Bot. 38, 498-505.

- Owen J.H., Laybourn-Parry J.E.M. and Wellburn A.R.
1986 Leaf respiration during early plastidogenesis in light-grown barley seedlings.
Physiologia Pl. 68, 100-106.
- Owen J.H., Laybourn-Parry J.E.M. and Wellburn A.R.
1987b Influences of light quality, growth regulations and inhibitors of protein synthesis on respiration of protoplasts from meristematic cells in barley seedlings.
Ann. Bot. 59, 99-105.
- Padgett R.A., Grabowski P.J., Konarska M.M., Seiler S. and Sharp P.A.
1986 Splicing of messenger RNA precursors.
Ann. Rev. Biochem. 55, 1119-1150.
- Padovan A.C., Dry I.B. and Wiskich J.T.
1989 Analysis of the control of phosphorylation coupled respiration in isolated plant mitochondria.
Plant Physiol. 90, 928-933.
- Palmer J.D., Jansen R.K., Michaels H.J., Chase M.W. and Manhart J.R.
1988 Chloroplast DNA variation and plant phylogeny.
Ann. Missouri Bot. Gard. 75, 1180-1206.
- Palmer J.M.
1976 The organisation and regulation of electron transport in plant mitochondria.
Ann. Rev. Plant Physiol. 27, 133-157.
- Palmer J.M. and Ward J.A.
1985 The oxidation of NADH by plant mitochondria.
In *Higher Plant Cell Respiration, Encyclopedia of Plant Physiology, New Series Vol 18*, eds R. Douce and D.A. Day, Springer-Verlag, Berlin, pp 173-201.
- Parikh V.S., Morgan M.M., Scott R., Clements L.S. and Butow R.A.
1987 The mitochondrial genotype can influence nuclear gene expression in yeast.
Science 235, 576-580.
- Parikh V.S., Webb H.C., Docherty R. and Butow R.A.
1989 Interaction between the yeast mitochondrial and nuclear genomes influences the abundance of novel transcripts derived from the spacer region of the nuclear ribosomal DNA repeat.
Mol. Cell Biol. 9, 1897-1907.

Pascal N, Dumas R. and Douce R.

1990 Comparison of the Kinetic behaviour toward pyridine nucleotides of NAD⁺-linked dehydrogenases from plant mitochondria.
Plant Physiol. 94, 189-193.

Pearson L. and Meagher R.B.

1990 Diverse soyabean actin transcripts contain a large intron in the untranslated leader: structural similarity to vertebrate muscle actin genes.
Plant Mol. Biol. 14, 513-526.

Pfanner N., Hoeben P., Tropschug M. and Neupert W.

1987 The carboxyl-terminus two thirds of the ADP/ATP carrier polypeptide contains sufficient information to direct translocation into mitochondria.
J. Biol. Chem. 262, 14851-14854.

Pfeifer K., Prezant T. and Guarente L.

1987 Yeast HAP1 activator binds to two upstream activation sites of different sequences.
Cell 49, 19-27.

Pike C.S. Kopecek K.K. Russell J.P. and Sceppa E.A.

1991 Regulation of protein phosphorylation in mitochondria of etiolated oat shoots.
Plant Physiol. Biochem. 29, 565-572.

Powell S.J., Medd S.M., Runswick M.J. and Walker J.E.

1989 Two bovine genes for mitochondrial ADP/ATP translocase expressed differently in various tissues.
Biochemistry 28, 866-873.

Pridmore R.D.

1987 New and versatile cloning vectors with kanamycin resistance marker.
Gene 56, 309-312.

Prioli L.M. and Söndahl M.R.

1989 Plant regeneration and recovery of fertile plants from protoplasts of maize (*Zea mays* L.)
Biotechnology 7, 589-594.

Prywes R., Dutta A., Cromlish J.A. and Roeder R.G.

1988 Phosphorylation of serum response factor, a factor that binds to the serum response element of the cFOS enhancer.
Proc. Natl. Acad. Sci. USA 85, 7206-7210.

Purdue P.E.

1988 *Nuclear genes and protein import into maize mitochondria.*
Ph.D. Thesis. University of Edinburgh.

Radloff R., Bauer W. and Vinograd J.

1967 A dye buoyant density method for the detection and isolation of closed circular DNA in HELA cells.
Proc. Natl. Acad. Sci. USA 57, 1515-1521.

Rawsthorne S., Hylton C.M., Smith A. and Woolhouse H.

1988 Photorespiratory metabolism and immunogold localisation of photo respiratory enzymes in leaves of C₃ and C₃-C₄ intermediate species of *Moricanda*.
Planta 173, 298-308.

Raymond P., Gidrol X., Salon C. and Pradet A.

1987 Control involving adenine and pyridine nucleotides.
In *The Biochemistry of Plants: A Comprehensive Treatise. Biochemistry of Metabolism*, ed D.D. Davies, 11, 129-176. New York: Academic.

Rebeille F., Bligny R. and Douce

1984 Is the cytosolic Pi concentration a limiting factor for plant cell respiration?
Plant Physiol. 74, 355-359.

Redinbaugh M.G., Sabre M. and Scandalios J.G.

1990 The distribution of catalase activity, isozyme protein, and transcript in the tissues of the developing maize seedling.
Plant Physiol. 92, 375-380.

Reed R. and Steitz J.A.

1985 Intron sequences involved in lariat formation during pre-mRNA splicing.
Cell 41, 95-105.

Reynolds G.A., Goldstein J.L. and Brown M.S.

1985 Multiple mRNAs for 3-Hydroxy-3-methylglutaryl Coenzyme A reductase determined by multiple transcription initiation sites and intron splicing sites in the 5' UTR.
J. Biol. Chem. 260, 10369-10377.

Rhodes M.J.C.

1980 Respiration and senescence of plant organs. In *The Biochemistry of Plants: A Comprehensive Treatise. Metabolism and Respiration*, ed D.D. Davies, 2, 419-462. New York, Academic.

Roberts J.K.M., Lane A.N., Clarke R.A. and Nieman R.H.

1985 Relationships between the rate of synthesis of ATP and the concentrations of reactants and products of ATP hydrolysis in maize root tips, determined by ^{31}P Nuclear Magnetic Resonance.
Arch. Biochem. Biophys. 240, 712-722.

Robinson D.C. and Wellburn A.R.

1981 Light quality and hormonal influences upon the rate of ATP formation by mitochondria during greening.
Biochem. Physiol. Pfl. 176, 54-59.

Roussel D.L., Thompson D.L., Pallardy S.G., Miles D. and Newton K.J.

1991 Chloroplast structure and function is altered in the NCS2 maize mitochondrial mutant.
Plant Physiol. 96, 232-238.

Runswick M.J., Powell, S.J., Nyren P. and Walker J.E.

1987/1987

Sequence of the bovine mitochondrial phosphate carrier protein: Structural relationship to ADP/ATP translocase and the brown fat mitochondria uncoupling protein.
EMBO J. 6, 1367-1373.

Runswick M.J., Walker J.E., Bisaccia F., Iacobazzi V. and Palmieri F.

1990 Sequence of the bovine 2-Oxoglutarate/malate carrier protein: Structural relationship to other mitochondrial transport proteins.
Biochemistry 29, 11033-11040.

Sachs M.M., Dennis E.S., Gerlach W.L. and Peacock W.J.

1986 Two alleles of maize alcohol dehydrogenase 1 have 3' structural and poly (A) addition polymorphisms.
Genetics 113, 449-467.

Saint-Guily A., Poh-Yam L., Chevalier C., Yamaguchi J. and Akazawa T.

1992 Unpublished but can be accessed from the EMBL, Genbank sequence database with the accession number X65549.

Sambrook J., Fritsch E.F. and Maniatis T.

1989 *Molecular Cloning*, Ed 2.

Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger F., Nicklen S. and Coulson A.R.

1977 DNA sequencing with chain terminating inhibitors.
Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Sarah C.J.

1991 *The import of proteins into isolated higher plant mitochondria.*

Ph.D. Thesis. University of Edinburgh.

Saraste M. and Walker J.E.

1982 Internal sequence repeats and the path of polypeptide in mitochondrial ADP/ATP translocase.

FEBS Letters. 144, 250-254.

Satler S.O. and Thimann K.V.

1983 Metabolism of oat leaves during senescence VII. The interaction of carbon dioxide and other atmospheric gases with light in controlling chlorophyll loss and senescence.

Plant Physiol. 71, 67-70.

Schmitz M.L., Maier U-G, Brown J.W.S and Feix G.

1989 Specific binding of nuclear proteins to the promoter region of a maize nuclear rRNA gene unit.

J. Biol. Chem. 264, 1467-1472.

Schultz J. and Carlson M.

1987 Molecular analysis of *SSN6*, a gene functionally related to the SNF1 protein kinase of *Saccharomyces cerevisiae*.

Mol. Cell Biol. 7, 3637-3645.

Schwarz-Sommer Z., Huijser P., Nacken W., Saedler H. and Sommer H

1990 Genetic control of flower development by homeotic genes in *Antirrhinum majus*.

Science 250, 931-936.

Senepathy P., Shapiro M.B. and Harris N.L.

1990 Splice junctions, branch point sites, and exons: Sequence statistics, identification, and applications to genome project.

Methods Enz. 183, 252-278.

Sharpe J.A. and Day A.

1992 Structure, evolution and expression of the mitochondrial ADP/ATP translocator gene from *Chlamydomonas reinhardtii*.

Submitted for publication.

Sheen J.

1990 Metabolic repression of transcription in higher plants.

Plant Cell 2, 1027-1038.

- Shillito R. and Harms C.T.
1989 Regeneration of fertile plants from protoplasts of elite inbred maize.
Biotechnology 7, 581-589.
- Short J.M., Fernandez, J.M., Sorge J.A. and Huse W.D.
1988 λ ZAP: A bacteriophage λ expression vector with in vivo excision properties.
Nucleic Acids Res. 16, 7583-7600.
- Simon E.W.
1984 Early events in germination.
In *Seed Physiology: Germination and Reserve Mobilisation*. ed. D.R. Murray, Academic Press, New York, pp. 77-115.
- Smagula C. and Douglas M.G.
1988 Mitochondrial import of the ADP/ATP carrier protein in *Saccharomyces cerevisiae*. Sequences required for receptor binding and membrane translocation.
J. Biol. Chem. 263, 6783-6790.
- Smith C.W.J., Patton J.G. and Nadal-Ginard B.
1989 Alternative splicing in the control of gene expression.
Ann. Rev. Genet. 23, 527-577.
- Sommarin M., Petit P.X. and Møller I.M.
1990 Endogenous protein phosphorylation in purified plant mitochondria.
Biochim. Biophys. Acta 1052, 195-203.
- Stephen D., Jones C. and Schofield J.P.
1990 A rapid method for isolating high quality plasmid DNA suitable for DNA sequencing.
Nucleic Acids Res. 18, 7463-7464.
- Stitt M., Lilley R. McC. and Heldt H.W.
1982 Adenine nucleotide levels in the cytosol, chloroplasts and mitochondria of wheat leaf protoplasts.
Plant Physiol. 70, 971-977.
- Stougaard J., Sandal N.N., Gron A., Kühle A. and Marcker K.A.
1987 5' Analysis of the soyabean leghaemoglobin lbc3 gene regulatory elements required for promoter activity and organ specificity.
EMBO J. 6, 3565-3570.

Suzuki H., Hosokawa Y., Nishikimi M. and Ozawa T.

1991 Existence of common homologous elements in the transcriptional regulatory regions of human nuclear genes and mitochondrial genes for the oxidative phosphorylation system.
J. Biol. Chem. 266, 2333-2338.

Szekely E and Montgomery D.L.

1984 Glucose represses transcription of *Saccharomyces cerevisiae* nuclear genes that encode mitochondrial components.
Mol. Cell Biol. 4, 939-946.

Tager J.M., Wanders R.J.A., Groen A.K., Kuntz W., Bohnensack R., Kuster U., Letko G., Bonme G., Duszynski J. and Wojtczack L.

1983 Control of mitochondrial respiration.
FEBS Letters. 151, 1-9.

Tang C.K. and Draper D.E.

1989 Unusual mRNA pseudoknot structure is recognised by a protein translational repressor.
Cell 57, 531-536.

Taylor W.C.

1989 Regulatory interactions between nuclear and plastid genomes.
Ann. Rev. Plant Physiol. Plant Mol. Biol. 40, 211-233.

Tetley R.M. and Thimann K.V.

1974 The metabolism of oat leaves during senescence. I. Respiration, carbohydrate metabolism and the action of cytokinins.
Plant Physiol. 54, 294-303.

Thomas D.R. and McNeil P.H.

1976 Effects of freezing on isolated plant mitochondria.
Planta 152, 242-247.

Thomas H. and Stoddart J.L.

1980 Leaf Senescence.
Ann. Rev. Plant Physiol. 31, 83-111.

Tokuhsa J.G., Singh K, Dennis E.S. and Peacock J.

1990 A DNA binding protein factor recognises two binding domains within the octopine synthase enhancer element.
Plant Cell 2, 215-224.

Tomura H., Endo H., Kagawa Y. and Ohta S.

1990 Novel regulatory enhancer in the nuclear gene of the human mitochondrial ATP synthase β subunit.
J. Biol. Chem. 265, 6525-6527.

Topping J.F.

1987 *Mitochondrial gene expression in the developing wheat leaf.*
Ph.D Thesis. University of Edinburgh.

Trawick J.D., Wright R.M. and Poyton R.O.

1989 Transcription of yeast COX6, the gene for the cytochrome *c* oxidase subunit VI, is dependant on heme and on the HAP2 gene.
J. Biol. Chem. 264, 7005-7008.

Trueblood C.E., Wright R.M. and Poyton R.O.

1988 Differential regulation of the two genes encoding *Saccharomyces cerevisiae* cytochrome *c* oxidase subunit V by heme and HAP2 and REO1 genes.
Mol. Cell Biol. 8, 4537-4540.

Uritani I. and Asahi T.

1980 Respiration and related metabolic activity in wounded and infected tissues.

In *The Biochemistry of Plants: A Comprehensive Treatise. Biochemistry of Metabolism*, ed D.D. Davies, 2, 463-485. New York: Academic.

Van der Ploeg L.H.T., Liu A.Y.C., Michels P.A.M. De Lange T., Borst P., Majumder H.K., Weber J., Veeneman G.H. and Van Boom J.

1982 Splicing is required to make the messenger RNA for a variant surface antigen in *Trypanosomes*.
Nucleic Acids Res. 10, 3591-3604.

Vasil V., Clancy M., Ferl R.J., Vasil I.K. and Hannah C.

1989 Increased gene expression by the first intron of maize shrunken-1 locus in grass species.
Plant Physiol. 91, 1575-1579.

Vignais P.V. and Satre M.

1984
Mol. Cell. Biochem. 60, 33-70.

von Heijne G.

1986 Mitochondrial targeting sequences may form amphiphilic helices.
EMBO J. 5, 1335-1342.

von Heijne G., Steppuhn J. and Herrmann R.G.

1989 Domain structure of mitochondrial and chloroplast targeting peptides.
Eur. J. Biochem. 180, 535-545.

Warmke H.E. and Lee S.L.-J.

1979 Organelle size and number in fertile and T-cytoplasmic male-sterile corn.

Amer. J. Bot. 66, 141-148.

Warmke H.E. and Lee S.L.-J.

1978 Pollen abortion in T cytoplasmic male-sterile corn (*Zea mays*): a suggested mechanism.

Science 200, 561-563.

Watson J.D., Hopkins N.H., Roberts J.W., Steitz J.A. and Weiner A.M.

1987 Regulation of protein synthesis and function in bacteria.

In *Molecular Biology of the Gene*. The Benjamin/Cummings Publishing Company Inc. Menlo Park, California. 4th Ed, pp 465-502.

Wedding R.T.

1989 Malic enzymes of higher plants. Characteristics, regulation and physiological function.

Plant Physiol. 90, 367-371.

Wellburn A.R. and Owen J.H.

1991 Control of the rate of respiration in shoots: light, calcium and plant growth regulators.

In *Compartmentation of Plant Metabolism in Non-Photosynthetic Tissues*, SEB Seminar Series Vol 42, ed M.J. Emes, Cambridge University Press, pp. 189-198.

Werr W., Frommer W.B. Maas C. and Starlinger P.

1985 The structure of the sucrose synthase gene on chromosome 9 of *Zea mays* L.

EMBO J. 4, 1373-1380.

Williams J.H.H. and Farrar J.F.

1990 Control of barley root respiration by sucrose.

Physiologia Pl. 79, 259-266.

Winkler H., Adam G., Mattes E., Schanz M., Hartig A. and Ruis H.

1988 Co-ordinate control of synthesis of mitochondrial and non-mitochondrial hemoproteins: A binding site for the HAP1 (CYP1) protein in the UAS region of the yeast catalase T gene (*CTT1*).

EMBO J. 7, 1799-1804.

Winning B.M., Bathgate B., Purdue P.E. and Leaver C.J.

1990 Nucleotide sequence of a cDNA encoding the beta subunit of the mitochondrial ATP synthase from *Zea mays*.

Nucleic Acids Res. 18, 5885.

Winning B.M., Sarah C.J., Purdue P.E., Day C.D. and Leaver C.J.

1992 The adenine nucleotide translocator of higher plants is synthesized as a large precursor that is processed upon import into mitochondria.

The Plant Journal 2, 763-773.

Winning B.W., Day C.D., Sarah C.J. and Leaver C.J.

1991 Nucleotide sequence of two cDNAs encoding the adenine nucleotide translocator from *Zea Mays* L.

Plant Mol. Biol. 17, 305-307.

Wiskich J.T. and Dry I.B.

1985 The tricarboxylic acid cycle in plant mitochondria: Its operation and regulation.

In *Higher Plant Cell Respiration, Encyclopedia of Plant Physiology, New Series* Vol 18, eds R. Douce and D.A. Day, Springer-Verlag, Berlin, pp. 281-313.

Wiskich J.T., Bryce J.H., Day D.A. and Dry I.B.

1990 Evidence for metabolic domains within the matrix compartment of pea leaf mitochondria.

Plant Physiol. 93, 611-616.

Wissinger B., Schuster W. and Brennicke A.

1991 Transplicing in *Oenothera* mitochondria: nad1 mRNAs are edited in exon and transplicing group II intron sequences.

Cell 65, 473-482.

Woodrow G. and Schatz G.

1979 The role of oxygen in the biosynthesis of cytochrome oxidase of yeast mitochondria.

J. Biol. Chem. 254, 6088-6093.

Wright R.M. and Poyton R.O.

1990 Release of two *Saccharomyces cerevisiae* cytochrome genes, COX6 and CYC1, from glucose repression requires SNF1 and SSN6 gene products.

Mol. Cell Biol. 10, 1297-1300.

Yanisch-Perron C., Vieira J. and Messing J.

1985 Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors.

Gene 33, 103-119.

Yemm E.W.

1965 The respiration of plants and their organs.

In *Plant Physiology. A treatise*, vol IV-A. ed F.C. Steward, Academic Press, New York, pp 231-310.

Yu S.M. and Kao C.H.

1981 Retardation of leaf senescence by inhibitors of RNA and protein synthesis.

Physiologia Pl. 52, 207-210.

APPENDIX

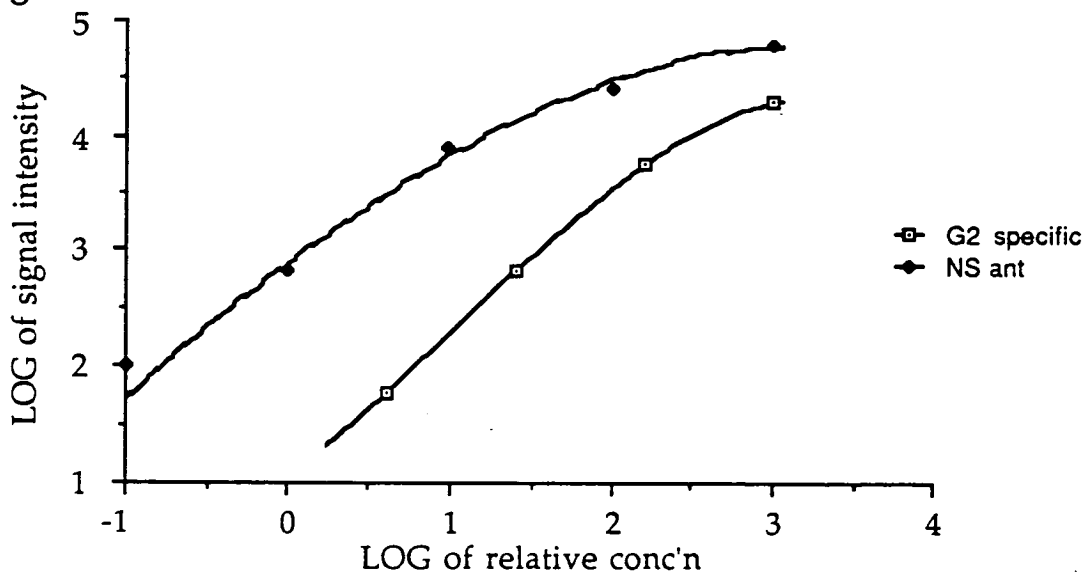
A I CALCULATING HYBRIDISATION SIGNAL RATIOS FOR NORTHERN BLOT ANALYSIS DATA

This section relates to the northern blots presented in figures 5.3, 5.4 and 5.6. The intensity of the hybridisation signals from each tissue was quantified using a densitometer by measuring the amount of exposure on each autoradiograph. To accurately determine the signal response curve of the film a control filter of a known dilution series was also exposed with each northern blot (Figure 5.4B). Data for the control dilution series in figure 5.4B is presented in table A.1 and figure A.1.

Table A.1

ANT2 (G2) control				NS ANT control			
SIGNAL INTENSITY	LOG	RELATIVE CONC'N	LOG	SIGNAL INTENSITY	LOG	RELATIVE CONC'N	LOG
19300	4.29	1000	5	65,200	4.81	1000	3
5690	3.76	160	4.2	28,200	4.45	100	2
677	2.83	25.6	3.41	8470	3.93	10	1
59.2	1.77	4.1	2.61	682	2.83	1	0
				112	2.05	0.1	-1

Figure A.1



It was observed that the signal response curve of the film is not linear. At high concentrations of sense RNA the film becomes over exposed due to an excess of radioactivity. For an accurate calculation of the ratio of hybridisation signals between the various tissues it is important to avoid over exposure of the film. A summary of the calculations to measure the ratios presented in figure 5.4A is below:

- 1) The intensity of the hybridisation signal in each tissue was calculated using a densitometer.
- 2) Using Figure A.1 the relative concentrations of the hybridisation signals were estimated by correlating the intensity of each hybridisation signal from the various tissues with the intensity of the control bands. This method of calculating the relative concentrations of each hybridisation signal should correct for errors due to the non-linear response of the film.
- 3) The concentrations of RNA used for northern blot analysis were normalised by measuring the level of EtBr staining of the maize ribosomal bands. The relative concentrations between the maize tissues studied, relative to the adventitious root tissue (which had the lowest amount of ribosomal RNA), were:

RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
1.3	1.1	1.2	1	1.2	1.1	1.2	1	1.3	1.3	1.1

- 4) Estimates for the intensity of the hybridisation signals and the relative rRNA concentrations between the tissues, has been calculated. Using this information it was possible to estimate the relative intensities of the hybridisation signals. The calculations are summarised in the tables A.2 and A.3. The signal intensity (SI) is used to estimate the relative concentration of the transcript levels by comparing it to the control standards in figure 5.4B and figure A.1. The relative transcript concentration in each tissue is then adjusted according to the amount of rRNA per sample. From this adjusted value (Rel. Conc'n (A)) the transcript ratios between the different tissues were calculated (Ratio of R.Conc'n)

Table A.2

NS ANT	RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
SI	4120	10800	10100	7100	3540	1150	2110	1280	3860	2250	4920
Log of SI	3.61	4.03	4.00	3.85	3.55	3.06	3.32	3.11	3.59	3.35	3.69
Log of R. Conc'n	0.65	1.17	1.12	0.92	0.59	0.16	0.38	0.20	0.63	0.40	0.73
Rel. Conc'n (A)	3.44	13.4	11.0	8.32	3.24	1.31	2.00	1.58	3.28	1.93	4.9
Ratio of R. Conc'n	2.5	10	8.5	6.5	2.5	1	1.5	1	2.5	1.5	3.5

Table A.3

MANT2 Specific	RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
SI	278	1250	960	495	434	185	240	179	482	219	615
Log of SI	2.44	3.10	2.98	2.69	2.64	2.27	2.38	2.25	2.68	2.34	2.79
Log of R. Conc'n	3.10	3.62	3.52	3.30	3.26	2.98	3.06	2.96	3.36	3.02	3.37
Rel. Conc'n (A)	968	3790	2760	2000	1520	868	957	912	1760	805	2130
Ratio of R. Conc'n	1	4.5	3	2.5	1.5	1	1	1	2	0.95	2.5

A II ATP SYNTHASE NORTHERN BLOTS

For the northern blots of the ATP synthase genes (see figure 5.6) the hybridisation signals were quantified using the densitometer. A control dilution series was not exposed with these northern blots but the autoradiograph was pre-flashed and developed in an identical manner to the autoradiographs above. An assumption was made that the autoradiograph response curve would be similar to those of the ANT autoradiographs in figure A.1. Therefore, the ANT2 (G2) control in figure A.1 and Table A.1 was used to estimate the relative concentrations using the same calculation as above (Tables A.2 and A.3). In the case of the mitochondrial genes which have a heterogeneous population of hybridisation signals the final relative concentrations for the upper (U) and lower (L) signals were calculated separately. Ratios based on these relative concentrations were calculated in addition to a ratio based on all the transcripts (T).

Table A.4

α subunit (U)	RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
SI	1580	2870	1920	944	1530	285	443	348	1320	965	2300
Log of SI	3.20	3.46	3.28	2.97	3.18	2.45	2.65	2.54	3.12	2.98	3.36
Log of R. Conc'n	3.70	3.90	3.76	3.51	3.68	3.11	3.26	3.18	3.63	3.52	3.82
Rel. Conc'n (A)	3860	7220	4800	3240	3400	1170	1520	1510	3280	2550	6010
Ratio of R. Conc'n	3.5	6	4	3	3.5	1	1.5	1.5	3	2	5
α subunit(L)(S)	5210	7780	2180	6900	4820	1630	3940	3900	660	4850	6510
Log of SI	3.72	3.89	3.34	3.83	3.68	3.21	3.60	3.59	2.82	3.69	3.81
Log of R. Conc'n	4.17	4.40	3.80	4.31	4.12	3.71	4.04	4.03	3.40	4.14	4.28
Rel. Conc'n (A)	11400	22800	5260	20400	11000	4660	9140	10700	1930	10600	17300
Ratio of R. Conc'n	2.5	5	1	4.5	2.5	1	2	2.5	0.4	2.5	3.5
α subunit T	2.5	5	1.5	4	2.5	1	2	2	0.9	2.5	4

Table A.5

ATP9 (U)	RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
SI	568	5210	3120	3060	1090	927	4120	2890	4310	2080	3250
Log of SI	2.75	3.72	3.49	3.48	3.04	2.97	3.61	3.46	3.63	3.32	3.51
Log of R. Conc'n	3.34	4.17	3.93	3.92	3.57	3.51	4.05	3.90	4.07	3.80	3.95
Rel. Conc'n (A)	1680	13400	7090	8320	3100	2940	9350	7940	9040	4850	8100
Ratio of R. Conc'n	0.55	4	2.5	3	1	1	3	2.5	3	1.5	3
ATP9 L (SI)	3010	5170	1970	4170	3480	3200	8800	5610	5690	4850	3210
Log of SI	3.48	3.71	3.29	3.62	3.54	3.51	3.94	3.75	3.76	3.69	3.51
Log of R. Conc'n	3.92	4.16	3.77	4.06	3.98	3.95	4.46	4.20	4.21	4.14	3.95
Rel. Conc'n (A)	6400	13100	4910	11500	7960	8100	24000	15800	12500	10600	8100
Ratio of Conc'n	0.8	1.5	0.6	1.5	1	1	3	2	1.5	1.5	1
ATP9 T	0.75	2.5	1	2	1	1	3	2	2	1.5	1.5

Table A.6

β subunit	RE	RT	ES	AR	S	L	K	CC	H4	H1	Si
SI	630	3120	2310	1370	619	577	497	403	877	454	1571
Log of SI	2.80	3.49	3.36	3.14	2.79	2.76	2.70	2.61	2.94	2.66	3.20
Log of R. Conc'n	3.38	3.93	3.82	3.64	3.37	3.34	3.30	3.24	3.49	3.28	3.70
Rel. Conc'n (A)	1845	7740	5510	4370	1950	1990	1660	1740	2340	1470	4560
Ratio of R. Conc'n	0.95	4	3	2	1	1	0.85	0.85	1	0.75	2.5
	1.5	5.5	3.5	3	1.5	1.5	1	1	1.5	1	3

A III THE SPECIFICITY OF VARIOUS ANT RNA PROBES USED FOR NORTHERN BLOT ANALYSIS

These tables refer to the data in figure 5.1.

Table A.7

<i>ANT1</i> specific					
pMANT1 SIGNAL INTENSITY		pMANT2 SIGNAL INTENSITY		RELATIVE CONC'N	
	LOG		LOG		LOG
7470	3.87	78.7	1.90	10,000	4
5920	3.77			2500	3.40
3020	3.48			625	2.80
1430	3.16			156	2.19
352	2.55			39	1.59
43.5	1.64			9.8	0.99

Table A.8

<i>ANT2</i> specific					
pMANT2 SIGNAL INTENSITY		pMANT1 SIGNAL INTENSITY		RELATIVE CONC'N	
	LOG		LOG		LOG
12600	4.10	32	1.51	10,000	4
6920	3.84			2500	3.40
3980	3.60			625	2.80
832	2.92			156	2.19
66	1.82			39	1.59
nd	nd			9.8	0.99

Table A.9

NS control					
pMANT1 SIGNAL INTENSITY LOG		pMANT2 SIGNAL INTENSITY LOG		RELATIVE CONCN LOG	
14900	4.17	16100	4.21	10,000	4
10200	4.01	14500	4.16	2500	3.40
6400	3.81	7850	3.89	625	2.80
2270	3.36	3520	3.55	156	2.19
543	2.73	835	2.92	39	1.59
84	1.92	168	2.23	9.8	0.99

Update section

Sequence

Nucleotide sequence of two cDNAs encoding the adenine nucleotide translocator from *Zea mays* L.

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The adenine nucleotide translocator (ANT), an inner mitochondrial membrane carrier protein, plays a central role in cellular energy metabolism, mediating the exchange of adenine nucleotides between the mitochondrial matrix and the cytosol. We have isolated two ANT cDNAs from maize (*Zea mays* L.), containing the entire coding sequences, with a view to gaining an understanding of the expression and biogenesis of this protein in higher plants. ANT cDNAs isolated previously in this laboratory were not full-length. One of these cDNAs, the insert from pANT1 [2], was used as a probe to screen a maize λ gt10 cDNA library. This library was constructed using cDNA synthesised from poly(A)⁺ RNA isolated from 3-day-old dark-grown seedlings. Two of the cDNAs isolated were found to correspond to the known ANT genes in maize, *G1* and *G2* [3]. It was found that the open reading frames of these cDNAs, designated MANT1 and MANT2, did not agree with the corresponding genomic clones. Therefore the genomic clones were resequenced

and it was found that sequencing errors had caused frameshifts; once these were corrected the open reading frames of the cDNAs and the genomic sequences were found to correspond exactly. Figure 1 shows a comparison of the nucleotide and deduced amino acid sequences of MANT1 and MANT2. The sequence analysis was carried out using the GAP computer program [4]. The predicted 387 amino acid proteins encoded by MANT1 and MANT2 show 98% similarity and are homologous to ANT proteins from fungal and mammalian sources [1, 5, 6] except that they contain N-terminal extensions. The significance of this will be discussed elsewhere (B. Winning, C.J. Sarah, C.D. Day and C.J. Leaver, in preparation).

Acknowledgments

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References

1. Arends H, Sebald W: Nucleotide sequence of the cloned mRNA and gene of the ADP/ATP carrier from *Neurospora crassa*. EMBO J 4: 2369–2376 (1984).
2. Baker A, Leaver CJ: Isolation and sequence of a cDNA encoding the adenine nucleotide translocator of *Zea mays* L. Nucl Acids Res 13: 5857–5867 (1985).
3. Bathgate B, Baker A, Leaver CJ: Two genes encode the adenine nucleotide translocator of maize mitochondria. Eur J Biochem 183: 303–310 (1989).
4. Devereux J, Haeberli P, Smithies O: A comprehensive set of sequence analysis programmes for the VAX. Nucl Acids Res 12: 387–395 (1984).
5. Lawson JE, Douglas MG: Separate genes encode functionally equivalent ADP/ATP carrier proteins in *Saccharomyces cerevisiae*. J Biol Chem 263: 14812–14818 (1988).
6. Neckelman N, Li K, Wade RP, Shuster R, Wallace DC: cDNA sequence of a human skeletal muscle ADP/ATP translocator: Lack of a leader peptide, divergene from a fibroblast translocator cDNA and coevolution with mitochondrial DNA genes. Proc Natl Acad Sci USA 84: 7580–7584 (1987).

Advances in Autotrophic Microbiology and One-Carbon Metabolism

Volume I

edited by **Geoff A. Codd; Lubbert Dijkhuizen; F. Robert Tabita**

ADVANCES IN AUTOTROPHIC MICROBIOLOGY AND ONE-CARBON METABOLISM 1

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1 CGCGCCTCTGAAAGTTCGGAGCGCGCGCCGCTCCGCTCCC...TCTCCGCTCCGCTCCCA...AGGCCAATAGTTTCAAGTGGCAATGGCGGAC 93
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A
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D Q L Q I L F F G K K Y G S G G A *
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1479 CGGGCAATTTTCTTACATATATGCTTTAAT 1511

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Fig. 1. Nucleotide and deduced amino acid sequence comparison of MANT1 and MANT2 cDNAs from *Zea mays* L. MANT1 is the upper sequence and the corresponding predicted amino acid sequence is shown above the nucleotide sequence. MANT2 is the lower sequence; in this case only the amino acid differences to the MANT1 primary sequence are shown below the nucleotide sequence. A putative polyadenylation signal, present in both cDNAs, is underlined.